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CHICKENPOX AND HERPES ZOSTER

II. OUCHTERLONY PRECIPITATION STUDIES

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THE gel-diffusion precipitation technique has not hitherto been used to study the relationship of chickenpox and zoster viruses, although the methods described by Oudin (1948) and Ouchterlony (1948) have been widely used in virus studies. Thus Jensen and Francis (1953) were the first to use both methods to indicate immunological differences and relationships between several strains of influenza virus and more recently Datt and Orlans (1958) found that a minor antigenic constituent common to both vaccinia and pig pox virus could be demonstrated only by agar diffusion techniques.

It seemed that the Ouchterlony agar diffusion technique might be of value in augmenting the results obtained by complement fixation in serological studies of chickenpox and zoster antisera and antigens (Taylor-Robinson and Downie, 1959). The results of such an investigation are given in this paper.

MATERIALS AND METHODS

Antigens.—Vesicle fluids from chickenpox and zoster patients were diluted at the time of collection from $\frac{3}{8}$ to $\frac{1}{8}$ with either phosphate-buffered saline, Hanks' solution, or a fluid containing 10 per cent horse serum, 10 per cent tryptic digest broth and 80 per cent Hanks' solution. The vesicle fluids were diluted in this way so that if required they could be used for tissue culture experiments. The tissue culture fluids were supernatant media from amnion cell cultures infected with chickenpox and zoster viruses. All fluids were stored at —70° in hard-glass tubes. Concentration of fluids of 5- and 25-fold was achieved by drying from the frozen state and reconstitution of the dried material in an appropriate volume of distilled water. Some fluids were freed from salt by dialysis against distilled water before concentration.

Sera.—The sera were those obtained from cases of chickenpox and zoster and previously used in complement-fixation tests (Taylor-Robinson and Downie, 1959). They were stored at —20° in hard-glass tubes and were not inactivated or diluted before use. In several indicated cases chickenpox sera concentrated fivefold were used. Sera to be concentrated were freed from lipid by centrifugation for 30 min. at 3,000 r.p.m., dried from the frozen state, and reconstituted in an appropriate volume of water.

Agar-diffusion technique.—The technique of Crumpton and Davies (1956) was followed except that the reservoirs were 5 mm. in diameter and their centres 8 mm. apart; they were punched out with a cork borer.

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RESULTS

Experiments using Vesicle Fluids as Antigen

Zoster vesicle fluid and zoster sera

Fig. 1 shows the reaction between 6 convalescent zoster sera and a zoster vesicle fluid. The lines of precipitation were discernible at 18 hr. and clearly visible after 48 hr. It can be seen that the lines of precipitation in one antigen-antibody system are contiguous with those of the neighbouring systems and that in at least 2 cases a minimum of 3 components can be identified. Similar line patterns were given by 21 convalescent zoster sera that were tested.

Sera collected from zoster cases during the first week of the rash were similarly tested against zoster vesicle fluid. Of 22 acute stage sera, 11 gave lines of precipitation, a two-component system being found in 3 cases and a one-component system in the other 8 cases.

Chickenpox vesicle fluid and zoster sera

Convalescent zoster sera gave lines of precipitation when tested also against a suitable chickenpox vesicle fluid and the reaction of chickenpox and zoster vesicle fluids with such sera is shown in Fig. 2. It can be seen that the lines of precipitation formed between convalescent zoster sera and the chickenpox vesicle fluid are continuous with those formed between these sera and zoster vesicle fluid. This finding suggests that with respect to convalescent zoster sera, chickenpox vesicle fluid contains the same antigens as zoster vesicle fluid. Four reservoirs in the experiment shown in Fig. 2 contained serum collected from 2 cases of chickenpox 11 weeks after onset; the sera failed to react with either antigen.

Vesicle fluids and convalescent chickenpox sera

In addition to the 2 chickenpox sera shown in Fig. 2, 21 convalescent chickenpox sera (collected between 22 and 25 days after the onset of the rash) were tested with zoster vesicle fluid. Lines of precipitation did not form. Chickenpox vesicle fluid was not available in sufficient quantity to test all the convalescent chickenpox

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EXPLANATION OF PLATES.

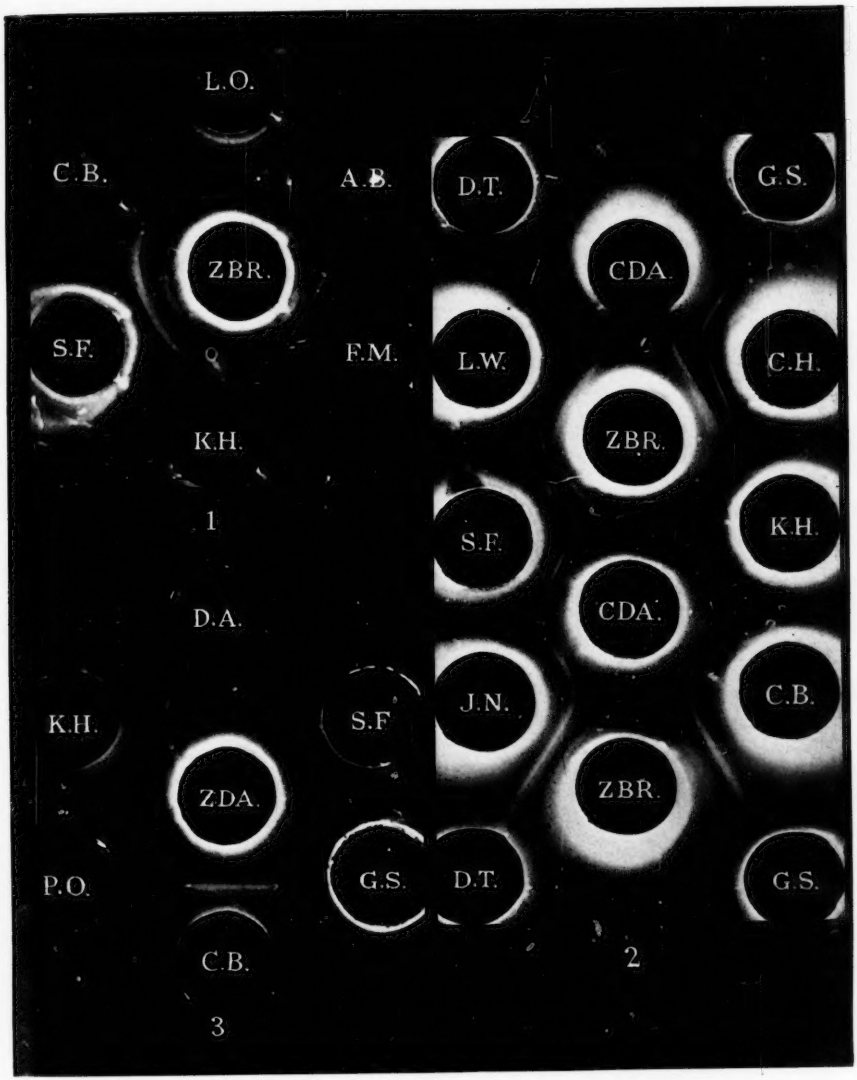
FIG. 1.—The reaction between convalescent zoster sera and zoster vesicle fluid.
Convalescent zoster sera: K.H., F.M., A.B., L.O., C.B., S.F. Zoster vesicle fluid: ZBR.

FIG. 2.—The reaction of convalescent zoster sera with zoster and chickenpox vesicle fluids.
Convalescent zoster sera: C.B., K.H., C.H., L.W., S.F., J.N. Zoster vesicle fluid: ZBR. Chickenpox vesicle fluid: CDA. D.T. and G.S. are unconcentrated convalescent chickenpox sera.

FIG. 3.—The reaction of zoster vesicle fluid with convalescent zoster sera and concentrated convalescent chickenpox sera.
Zoster vesicle fluid: ZDA. Convalescent zoster sera: C.B., S.F., K.H. Concentrated convalescent chickenpox sera: G.S., D.A., P.O.

FIG. 4.—The reaction of convalescent zoster sera and concentrated convalescent chickenpox sera with zoster and chickenpox vesicle fluid.
Convalescent zoster sera: K.H., L.O., A.B. Concentrated convalescent chickenpox sera: D.A., A.P., P.O. Zoster vesicle fluid ZM. Chickenpox vesicle fluid CW.

FIG. 5.—The reaction between a concentrated zoster tissue culture fluid and convalescent zoster sera.
Concentrated zoster tissue culture fluid: ZG. Convalescent zoster sera: C.B., M.M., C.H., C[illegible], K.H. G.S. is an unconcentrated chickenpox serum.



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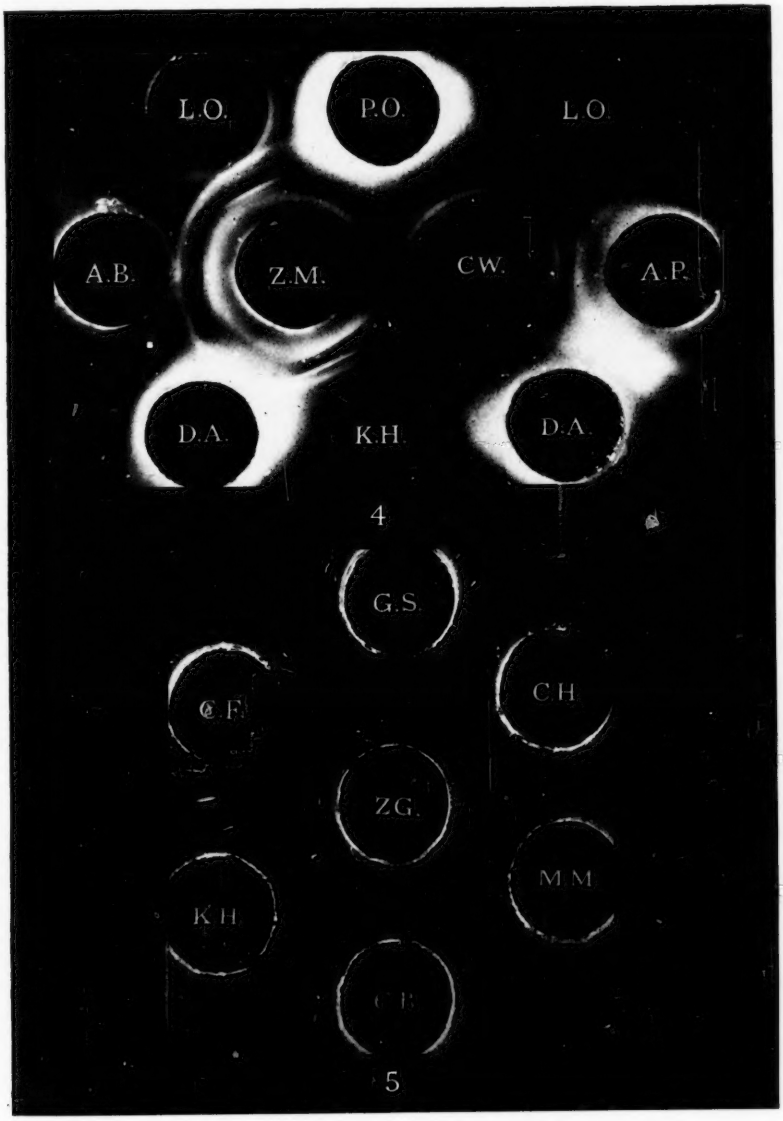

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sera, but 2 chickenpox fluids which gave marked lines of precipitation with convalescent zoster sera were tested against 8 convalescent chickenpox sera. Four of these sera at dilutions of $\frac{1}{4}$ or $\frac{1}{8}$ fixed complement with standard zoster antigen but none of the sera gave lines of precipitation with chickenpox vesicle fluid.

It seemed possible that the failure of convalescent chickenpox sera to produce lines of precipitation with vesicle fluids giving satisfactory line patterns with zoster sera might be due to insufficient concentration of antibody in the chickenpox sera. Three chickenpox sera concentrated fivefold and 3 unconcentrated zoster sera were tested therefore against a zoster vesicle fluid. The result is shown in Fig. 3. It is apparent that, although the chickenpox sera react less strongly than the zoster sera, the lines of precipitation formed by the different sera are continuous, suggesting that the precipitating antibodies demonstrable by concentration of the chickenpox sera are similar to those present in zoster sera. Fig. 4 shows the reaction of convalescent zoster and concentrated convalescent chickenpox sera with zoster and chickenpox vesicle fluids. Although the reactions with the chickenpox fluid are rather weak, they do not indicate any qualitative difference between the antigens in vesicle fluids from chickenpox and zoster cases.

Experiments Using Tissue Culture Fluid as Antigen

Doubling dilutions of vesicle fluids were tested against undiluted zoster convalescent sera. It was found that good fluids gave lines of precipitation up to a dilution of 1/6 but not at 1/12. The same antigens fixed complement with convalescent zoster sera (diluted $\frac{1}{4}$ or $\frac{1}{8}$) up to dilutions of 1/80 or even 1/160. It appeared therefore that with the techniques employed, a 10- to 20-fold greater concentration of vesicle fluid was necessary for the demonstration of precipitation than would suffice to fix complement.

Tissue culture fluids which fixed complement did so only when undiluted. It was not to be expected therefore that such fluids would give precipitation with convalescent sera and even after 5-fold concentration lines of precipitation were not obtained. Tissue culture fluids which were shown to fix complement undiluted were found, however, to give line patterns when concentrated 25 times. Fig. 5 shows the reaction obtained between a twenty-five fold concentrated zoster tissue culture fluid and 5 convalescent zoster sera. The sixth serum was from an early case of chickenpox.

In a further experiment concentrated zoster and chickenpox tissue culture fluids were tested against 10 convalescent zoster sera and 2 concentrated convalescent chickenpox sera. Lines of precipitation were observed which appeared to be continuous between the various sera and the 2 tissue culture fluids. The line pattern was unsuitable for photographic reproduction however, being obscured by non-specific zones of opacity around the antigen reservoirs.

The Complement-fixing and Precipitating Systems

All the sera examined by the agar diffusion technique had been tested by complement fixation. A comparison of the results observed with convalescent zoster sera showed a rough parallelism in that those sera possessing a high complement-fixing titre usually gave more sharply defined lines of precipitation than those exhibiting a low complement-fixing titre. Of 22 acute zoster sera, collected

within 7 days of the onset of the rash, 11 gave lines of precipitation with zoster vesicle fluids; these reactions were adjudged weaker than those produced by convalescent sera from the same patients. Of these 11 sera, only 7 fixed complement in dilutions of $\frac{1}{4}$ or higher. Of the 11 acute zoster sera which failed to give lines of precipitation, one fixed complement at a serum dilution of $\frac{1}{4}$. On the other hand, of 21 convalescent chickenpox sera none gave lines of precipitation when tested against zoster vesicle fluid known to contain precipitable antigen, although 8 of them fixed complement in a serum dilution of $\frac{1}{4}$ or $\frac{1}{8}$. Similarly 8 convalescent chickenpox sera failed to precipitate with a chickenpox vesicle fluid which gave well marked lines of precipitation with zoster sera although 3 or these 8 sera fixed complement with zoster or chickenpox vesicle fluid. Moreover of 2 zoster vesicle fluids one had a complement-fixing titre of 1/40 and gave lines of precipitation at a dilution of $\frac{1}{6}$ while the other fluid had a complement-fixing titre of 1/80 and gave lines of precipitation when diluted $\frac{1}{3}$ but not $\frac{1}{6}$. The zoster serum was used at a dilution of $\frac{1}{8}$ in the complement fixation tests and was used undiluted in the agar diffusion experiments. The antigen-antibody reactions apparent in agar diffusion tests are not necessarily the only reactions on which fixation of complement depends and strict parallelism of the results from the two techniques is not, perhaps, to be expected.

SUMMARY

In agar diffusion experiments lines of precipitation were observed between zoster vesicle fluids and 21 convalescent zoster sera. Line patterns could be resolved into at least 3 components, presumably representing at least 3 antigen-antibody pairs in the zoster-antizoster precipitating system.

Chickenpox vesicle fluid also produced lines of precipitation with convalescent zoster sera. These lines fused with those produced by the same sera and a zoster vesicle fluid indicating the identity of the reacting antigens in the two fluids.

With convalescent zoster and concentrated convalescent chickenpox sera, tissue culture fluids produced lines of precipitation only when concentrated 25-fold.

Twenty-two convalescent chickenpox sera failed to produce lines of precipitation with a zoster vesicle fluid. On five-fold concentration, however, 6 sera tested gave lines of precipitation with zoster and chickenpox vesicle fluids which fused with the lines of precipitation given by these fluids and convalescent zoster sera.

The results of this investigation failed to reveal differences in the precipitable antigens of zoster and chickenpox vesicle fluids and the precipitating antibodies in zoster and chickenpox sera, although it appeared that convalescent chickenpox sera had a much lower concentration of antibody than zoster sera. These findings are not unexpected if one accepts the view that zoster is in most cases a clinical expression of the reinvasion of tissues by a latent chickenpox virus.

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CHICKENPOX AND HERPES ZOSTER
III. TISSUE CULTURE STUDIES

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SERIAL propagation of virus from chickenpox or zoster cases has not been accomplished in laboratory animals, including the chick embryo, although the virus from zoster cases has been found to produce typical intranuclear inclusions in small pieces of human skin grafted on the chorio-allantois (Goodpasture and Anderson, 1944 ; Blank, Coriell and Scott, 1948 ; Caunt, personal communication).

In 1952, Weller and Stoddard reported the occurrence of eosinophilic intranuclear inclusions in cultures of tissue fragments of human embryo infected with chickenpox vesicle fluid. In a subsequent paper (Weller, 1953) it was reported that virus from several cases of chickenpox and herpes zoster had been propagated in roller tube cultures of human embryonic tissue ; but subculture was only successful when the inoculum contained infected cells. The cytological changes in the cultures inoculated with zoster vesicle fluid were indistinguishable from those produced by chickenpox vesicle fluid. Inhibition of the cytopathic effects of the virus in tissue culture was effected equally by convalescent zoster or chickenpox sera (Weller, 1958 ; Weller and Witton, 1958).

In this paper, experiments on the cultivation of viruses from chickenpox and zoster vesicle fluid in tissue cultures of human fibroblasts and of amnion cells are described ; and neutralization tests have been made which, like those of Weller, lend support to the view that viruses from chickenpox and zoster are immunologically identical.

MATERIALS AND METHODS

Human fibroblast cells

Fragment cultures in tubes were prepared by the method described by Feller *et al.* (1940). They were incubated on a roller drum. Human tissue, from 12–20 weeks-old embryos removed by hysterotomy, and human foreskin were used.

With embryonic fragments fibroblastic outgrowth was first seen on the 2nd day and by the 4th–5th day there was usually a luxuriant network of fibroblast cells. To maintain the cultures it was necessary to change the medium approximately every 3 to 5 days. With foreskin fragment cultures fibroblastic outgrowths appeared on the 3rd–5th day and the growth was not suitable for virus inoculation until the 10th–14th day. For these, it was necessary to change the medium about once every week.

Trypsin-dispersed cultures of human foreskin were prepared according to the method of Youngner (1954). Usually three foreskins were digested for 45–60 min. with 0·25 per cent " Difco trypsin 1:250 " made up in phosphate-buffered saline. The resulting cell suspension was removed and the extraction repeated with fresh trypsin 3 or 4 times. The cells in the pooled extracts were deposited by low speed centrifugation and resuspended in culture medium. The final cell suspension containing 5–10 $\times$ 10^5 cells per ml. was distributed in 1 ml. amounts to 6 $\times$ $\frac{5}{8}$ in. Pyrex rimless test tubes. A complete sheet of cells formed in

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about 6 days. The medium was changed at least once a week. Cell suspensions for subculture were prepared by using 0·25 per cent trypsin; in this way a "line" of human foreskin fibroblasts was maintained through 20 passages over a period of 26 weeks. All cultures prepared by trypsin dispersion of cells were kept stationary during incubation.

Human amnion cells

The successful growth of human amnion cells in a monolayer culture was first described by Zitcer *et al.* (1955) and their technique has usually been followed. The number of trypsin extractions of amnion was usually 5 or 6, the first of which was discarded. The final cell suspension was made up to contain 3–4 $\times$ 10^5 cells per ml. The cells were distributed in 1 ml. amounts to 6 $\times$ $\frac{5}{8}$ in. Pyrex test tubes and sometimes to flat-sided "medicine" bottles. For 6 oz., 4 oz. and 2 oz. bottles, 20, 14 and 5 ml. of cell suspension respectively were used. Each amnion provided sufficient cell suspension for 200–400 culture tubes. A change of medium on the 4th–6th day removed loose cells and accelerated the formation of a confluent cell sheet which was usually attained on the 7th–14th day. The medium was changed thereafter about once a week. Cultures sometimes began to degenerate after only 10–14 days, but in many instances cultures remained in good condition for 4 weeks or longer. Fibroblast-like cells sometimes appeared when the medium contained a high proportion of serum. This was especially liable to occur with a concentration of human serum greater than 20 per cent and was a reversible change.

Attempts to produce a "line" of amnion cells, as described by Fogh and Lund (1957), were not successful. Cells removed from bottles by trypsin never produced the confluent sheets in tubes shown by primary cultures. Hence subcultures of amnion cells were not employed.

Because focal lesions in amnion cell sheets tended to be more circumscribed and consequently easier to count than those in fibroblast cultures, amnion cells were used in most of the experiments recorded below.

Media

Initially, human fibroblast cells were grown in a medium containing human serum, for example:— 10 per cent inactivated human serum, 10 per cent inactivated horse serum, 5 per cent lactalbumen hydrolysate and 75 per cent Hanks' solution. When cultures were well established, the human serum was omitted from the medium.

An example of the type of medium in which human amnion cells were initially grown is as follows:— 10 per cent inactivated horse serum, 10 per cent tryptic digest broth, 5 per cent lactalbumen hydrolysate, 20 per cent bovine amniotic fluid and 55 per cent Hanks' solution. Sometimes the lactalbumen hydrolysate and the bovine amniotic fluid were omitted and other minor variations in the proportions of constitutents were made.

Inoculation of cultures

The inoculum consisted of vesicle fluid collected on the first few days of the eruption from chickenpox and zoster patients. The fluid was diluted immediately with 1·0 ml. of Hanks' solution or culture medium to prevent coagulation and, if not inoculated directly into tissue cultures, was stored at —70°. Before inoculation the medium was removed from tubes showing satisfactory cell growth and 0·1 ml. of diluted vesicle fluid added to each. After about 30 min. medium was added and the tubes incubated again at 37°.

Sera used in neutralization tests

Acute and convalescent sera from chickenpox and zoster cases were the same as those used in the complement-fixation and precipitation tests described previously. They were inactivated at 57° for 20 min. before use.

RESULTS

Cytopathic changes in cultures of human fibroblast and amnion cells

The nature and progress of lesions in chickenpox-infected cultures were never distinguishable from those in zoster-infected cultures, and the following description applies equally to both.

In sheets of fibroblast cells focal cytopathic changes were usually first observed after 4–6 days. These changes consisted in the formation of rounded, refractile cells. The lesions slowly increased in size by peripheral involvement of contiguous cells as described by Weller (1953) and Weller, Witton and Bell (1958).

In amnion cell sheets, again the first change, noted after 4 or 5 days, was a focal one. Cells became slightly more refractile but were not, at first, separated from their neighbours. By use of the collodion technique (Cheatham, 1954) sheets of cells were removed from the tubes and stained with haematoxylin and eosin. Infected cells contained eosinophilic intranuclear inclusion bodies as shown in Fig. 1. Multinucleated cells were frequently seen and in these each nucleus usually contained an inclusion (Fig. 2). Further incubation resulted in the " rounding up " and detachment of cells from one another. Again the lesions enlarged by the involvement of contiguous cells and the central cells became granular and necrotic. In a compact cell sheet the lesions were roughly circular (Fig. 3) rather than elongated as in fibroblast cultures. Eventually an amnion cell sheet would become completely infected and degenerate.

In one strain from chickenpox (CDT.) which has been passed repeatedly, a change in the character of cytopathic effects was noted in the 14th passage and has persisted in subsequent subcultures. In addition to the changes already described there were large syncitial masses with inclusion-containing nuclei gathered together in groups. This appearance was observed by Weller, Witton and Bell (1958), particularly in cells of epithelial origin.

The isolation and subculture of chickenpox and zoster virus strains in tissue culture

As observed by Weller (1953) the continued passage of virus strains in tissue culture was only successful if the inoculum contained infected tissue cells. At first cells were scraped off the walls of culture tubes which showed typical lesions and the resultant suspension of cells used as inoculum for fresh culture tubes. However, it was found that if cells from infected cultures were detached from the tubes and dispersed by the use of trypsin, the number of lesions produced on subculture could be greatly increased. The trypsinized cell suspension was centrifuged at 2,000 r.p.m. for 10 min. (625 *g*) and the deposit thoroughly dispersed in culture medium for inoculation into fresh cultures.

The strains of virus isolated and information about the time of appearance of lesions and subculture are shown in Table I. Virus was not grown from chickenpox vesicle fluid taken on the 4th day of the rash or later. From zoster cases vesicle fluid was occasionally infective as late as the 6th day from the onset of the rash. This difference may be due, in part, to the fact that zoster fluid was usually obtainable in greater amount so that cultures received a relatively more concentrated inoculum. There were no distinguishing morphological features among the 22 strains of chickenpox and the 21 strains of zoster. From one patient (ZWR.) virus was grown from the zoster vesicles and from the accompanying generalized varicelliform eruption. The earlier changes (increased refractility of cells) might be seen after 2 days, especially on subculture, although more often focal lesions were first detected after 4 to 6 days. Subculture was only attempted with some of the strains and these were usually only transferred through a few passages. One strain from chickenpox (CDT.) has been carried through 33 subcultures over a period of 22 months.

TABLE I.—*Strains of Chickenpox and Zoster Virus Isolated in Tissue Culture and their Continued Passage*

Chickenpox strains	Duration of rash in days when vesicle fluid taken	Days in culture before lesions first noted	Number of passages in tissue culture
CPL.	1	4	3
CLI.	1	4	0
CWI.	1	4	0
CZW.	1	5	0
CBU.	1	4	1
CIS.	1	5	1
CWO.	1	?	1
CAP.	1	6	0
CNI.	2	6	5
CPE.	2	7	0
CHO.	2	4	1
CFU.	2	4	1
CBL.	2	?	2
COB.	2	3	0
CDT.	2	5	33
CTH.	2	4	0
	4	—	0
CGS.	2	6	2
	3	6	1
	4	—	0
CDA.	3	4	1
CCO.	3	4	2
CPI.	3	9	5
CBI.	3	4	0
CUN.	3	6	1
	4	—	0
	5	—	0
Zoster strains			
ZTH.	1	4	1
ZTO.	2	2	0
ZTA.	1	4	2
ZBR.	2	4	1
ZHA.	2	3	1
	3	5	1
ZPE.	2	4	1
ZAB.	3	4	7
ZLO.	3	6	0
ZRO.	3	4	3
ZMA.	3	5	1
ZGU.	3	7	0
ZLA.	3	7	1
ZJO.	3	?	3
ZLY.	3	5	0
ZWH.	4	5	2
ZMO.	4	8	1
ZFO.	4	6	1
ZNE.	5	?	3
ZWR.	6	7	0
ZWA.	6	9 (2 lesions)	0
ZBO.	6	3	0

? = not recorded.
— = no focal lesions seen.
0 = strain of virus not subcultured.

The absence of virus from the fluid phase of tissue cultures

It was frequently observed in tissue cultures kept stationary that there was an increase in the number of lesions during the first 10 days. But if there were only 1 or 2 lesions at the end of this time, further lesions did not appear and progressive infection of the amnion sheet occurred through peripheral extension of the existing lesions. This suggested that there was no seeding of free virus to the healthy cells at a distance from the lesions.

On many occasions during the course of routine subculture, medium from infected culture tubes was removed, centrifuged at 2,000 r.p.m. (625 *g*) for 10 min. and the supernatant fluid inoculated onto fresh tissue cultures. Focal lesions never resulted. In one experiment, however, the medium from a bottle culture showing very gross cytopathic changes, was centrifuged at 2,000 r.p.m. for 10 min. (625 *g*). In 4 tissue culture tubes 5, 3, 5 and 4 lesions resulted from the inoculation of 1 ml. quantities of the supernatant fluid.

Weller (1958) reported that one virus strain of cytomegalic inclusion disease showed adaptation to tissue culture conditions, in that cell-free virus was demonstrated in the 3rd subculture. The chickenpox strain CDT. through 33 passages has not shown a similar adaptation nor has such been observed by Weller, Witton and Bell (1958) in their chickenpox-zoster strains.

Weller (1958) failed to release infective virus from tissue culture cells by various procedures designed to disrupt the cells. A similar failure has been encountered in these studies although a small amount of free infective virus has, on occasion, been obtained by using ultrasonic methods. These and other experiments concerning the close relationship between virus and tissue culture cells will be reported elsewhere.

The presence of free virus in vesicle fluid

Centrifugation experiments with zoster vesicle fluid suggested that extracellular virus was present in the fluid. A small sample (0·1 ml.) of vesicle fluid was diluted in 3 ml. of tissue culture medium and centrifuged at 3,000 r.p.m. (1400*g*) for 20 min. The top 1 ml. was carefully removed and added to a tissue culture tube. Similarly the next 1 ml. was examined and the deposit, resuspended in the last 1 ml. of fluid, was inoculated into a third culture tube. After 7 days the 3 culture tubes showed 70, 150 and several hundred lesions respectively. To demonstrate that this result was not due to the presence of cells or cell fragments in the supernatant fluid the following filtration experiment was carried out :—

Zoster vesicle fluid (0·3 ml.) taken from a patient on the 3rd day of the rash, was diluted with tissue culture medium to 4·0 ml. and 1·0 ml. was inoculated immediately into a culture of amnion cells. The remaining 3·0 ml. was filtered through a Gradocol filter of pore size 0·81 mμ. A positive pressure of 1–2 lbs./sq. in. was used and the filtrate divided between 3 amnion culture tubes, which were incubated at 37° for 6 days. The unfiltered fluid produced 80 focal lesions and the 3 tubes inoculated with filtrate showed 36, 18 and 14 lesions respectively. The filter was shown to be impervious to a culture of *Chromobacterium prodigiosum*. This experiment demonstrated unequivocally the presence of extra-cellular infective virus. Similar experiments were not performed with chickenpox vesicle fluid because of the lack of this material.

The effect of temperature on vesicle fluids and infected tissue culture cells

Vesicle fluids stored at −70° have been found infective after 21 months, the longest period tested. The fall in infective titre in one zoster fluid stored at −70° for 75 days was less than 50 per cent. On the other hand, in several experiments infected tissue culture cells were no longer infective after 24 hours at this temperature, although at 18° infectivity was maintained for several days. It would seem that the infectivity of chickenpox and zoster vesicle fluids stored at −70° is dependent upon the existence of free virus therein. Virus within tissue culture cells, perhaps in an immature form, seems to be inactivated with the death of the cell, as was suggested by Weller *et al.* (1958).

Neutralization tests

In neutralization tests in tissue culture Weller and Witton (1958) used as their source of virus suspensions of infected tissue culture cells. They found that incubation of a suspension of infected cells with antibody-containing serum resulted in little inhibition of focal cytopathogenicity when the mixture was transferred to susceptible tissue cultures. However, the incorporation of the serum as an integral part of the medium of the cultures had an inhibitory effect. In the presence of convalescent serum there was a delay in the appearance of focal lesions and a significant reduction in their number. Chickenpox and zoster strains behaved similarly in neutralization tests in the presence of sera derived from either disease. In no instance was complete neutralization observed.

Tests with vesicle fluid as a source of virus

Because free virus had been demonstrated in zoster vesicle fluid it seemed that vesicle fluids would be of greater value than infected cell suspensions in which virus within the cell is presumably protected from the effects of immune serum.

The following technique was used initially:— approximately 0·5 ml. of zoster vesicle fluid (ZTA.) obtained on the first day of the rash, was mixed with 27·5 ml. of tissue culture medium (without horse serum). In 3 × ½ in. tubes, 0·8 ml. quantities of the diluted vesicle fluid were then mixed with 0·2 ml. quantities of inactivated serum, acute or convalescent, from chickenpox or zoster patients. The tubes were shaken thoroughly and left at room temperature for 1½ hr. The mixtures were then inoculated into tubes of amnion culture from which the medium

EXPLANATION OF PLATES

FIG. 1.—An early focal lesion of infected amnion cells 5 days after inoculation with zoster vesicle fluid. Note intranuclear inclusions. H. & E. × 290.

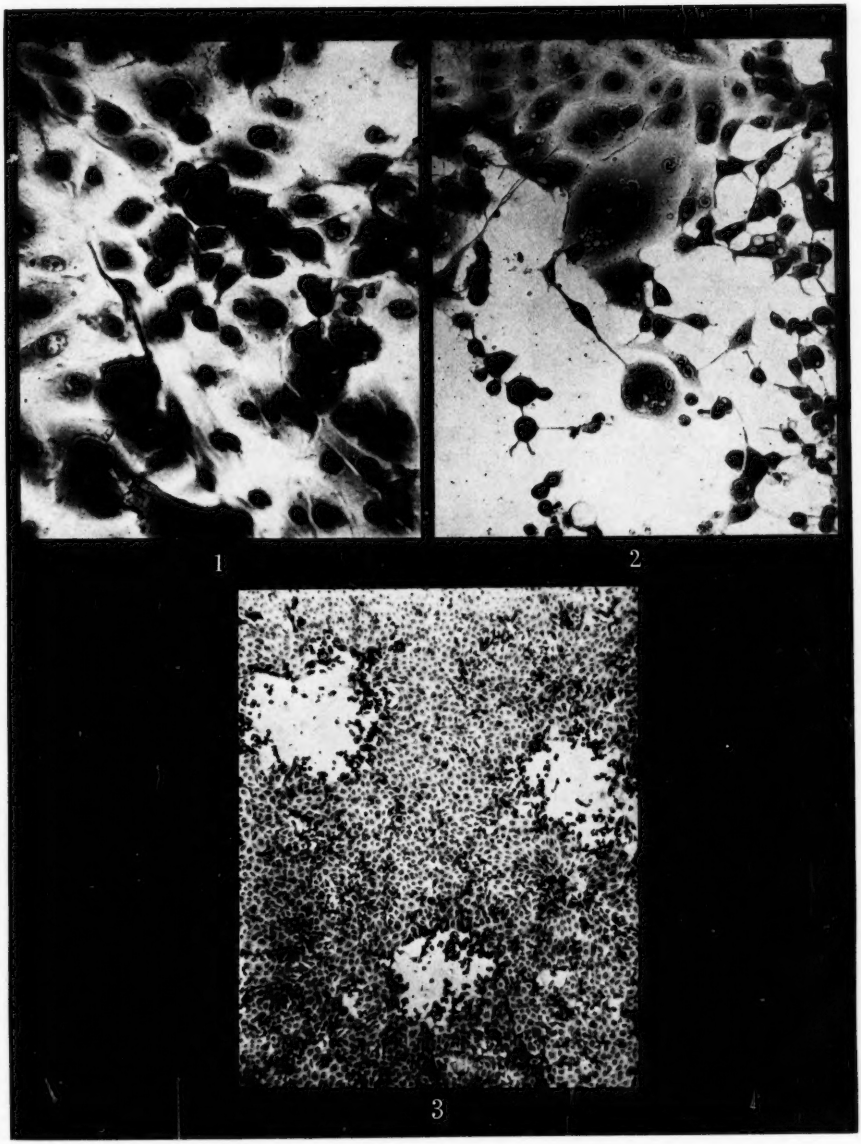
FIG. 2.—Typical multinucleated giant cells, each nucleus containing an inclusion body, in an amnion cell culture infected with chickenpox vesicle fluid. H. & E. × 240.

FIG. 3.—A compact sheet of amnion cells showing 3 focal lesions. The degenerate cells in the centre of the lesions have been lost in making the preparation. H. & E. × 29.

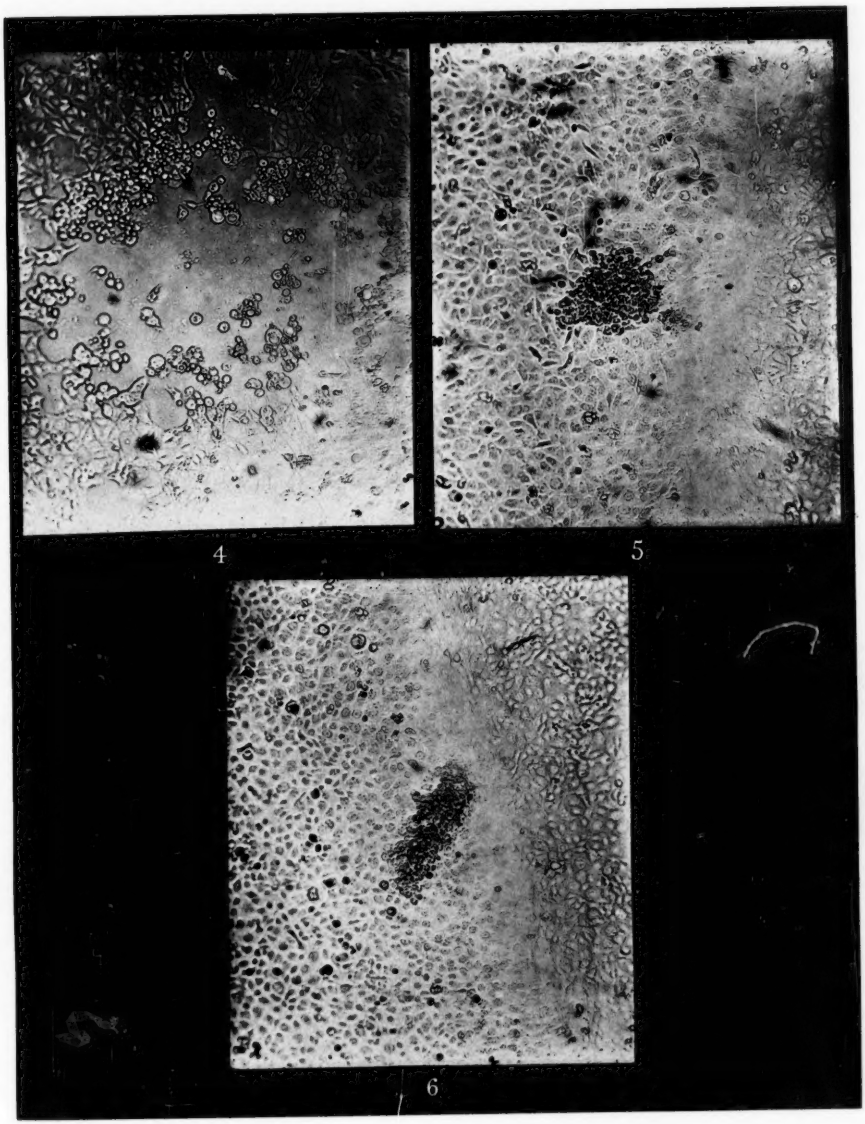
FIG. 4.—A neutralization test with zoster-infected cells as virus inoculum. The medium in this control amnion culture tube contained 5 per cent acute chickenpox serum. Note: cells photographed in the tube. × 36.

FIG. 5.—The same neutralization test as in Fig. 4 but the medium contained 5 per cent convalescent chickenpox serum (W.E.). Note the restriction in size of the lesion. × 36.

FIG. 6.—The same neutralization test as in Fig. 4 but the medium contained 5 per cent convalescent zoster serum (E.F.). Again there is marked restriction in size of the lesion. × 36.



Taylor-Robinson.



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had been removed. Each tube then contained 20 per cent human serum. Two culture tubes were used for each serum tested. Four control tubes containing 20 per cent horse serum in place of the human serum were also set up. After incubation at 37° for 5 days the number of focal lesions in each tube was counted. The results are shown in Table II. Unfortunately a control human serum, from a child known not to have had chickenpox, caused non-specific degeneration of the amnion cells.

TABLE II.—*The Result of a Neutralization Test Using Zoster Vesicle Fluid (ZTA.)*

Patient	Duration of rash when serum taken (days)	Number of focal lesions in each of two culture tubes
		Zoster sera
C. B—	2	0, 1
	23	0, 0
S. F—	3	0, 4
	24	0, 0
C. H—	3	20, 10
	25	0, 0
		Chickenpox sera
W. I—	2	96, 100
	25	0, 1
W. E—	1	168, 176
	24	0, 0
W. H—	2	68, 70
	25	12, 10
I. T—	2	268, 180
	23	0, 0
Control tubes.	Horse serum only	700*, 650*, 600*, 800*

*Only very approximate count because of the number of lesions.

It may be seen that there is a very marked reduction in the number of focal lesions even with acute chickenpox serum. Because of the absence of the control with non-immune human serum, it is uncertain whether this result was due to the non-specific effect of human serum or to its antibody content or indeed the result of both. However, it would seem that acute zoster sera show greater neutralizing activity than chickenpox sera taken at the same interval after the onset of the rash. Both chickenpox and zoster convalescent sera at this dilution produced almost complete neutralization. Similar results were obtained in a further experiment with another zoster vesicle fluid.

In subsequent tests a simpler technique was used. Inactivated sera in 0·05 ml. quantities were placed in tube cultures of amnion cells from which the medium had been removed. Zoster vesicle fluid (ZTO.), diluted with Hanks' solution, was then added in 0·05 ml. quantities to each tube. The tubes were rocked 30 times to ensure mixing and left at room temperature for 30 min. Then 0·9 ml. of tissue culture medium (without horse serum) was added to each to give a final concentration of 5 per cent human serum. The tubes were again rocked several times, and incubated at 37°. Focal lesions were counted after 4 and 8 days; the results are shown in Table III. Again there is good cross neutralization. Similar results were obtained in another test made in this way with a zoster vesicle fluid.

TABLE III.—*The Result of a Neutralization Test Using Zoster Vesicle Fluid (ZTO.)*

Patient	Duration of rash when serum taken (days)	Number of focal lesions in each of two culture tubes: Result on 4th day	Result on 8th day
		Zoster sera	
S. A—	4	0, 0	4, 1
	25	0, 1	0, 2
		Chickenpox sera	
S. D—	2	28, 42	100, 110
	23	1, 0	2, 4
W. I—	2	27, 22	94, 80
	25	0, 0	1, 1
J. J. B—	3	38, 70	85, 100+
	120	0, 1	3, 2
Control tubes.	Horse serum only	40, 44	100+, 100+

Because only small amounts of vesicle fluid could be obtained, sera were not tested in serial dilutions. Nevertheless, the reduction in the number of focal lesions is such that the results are obviously significant. In all, 7 paired zoster sera and 9 paired chickenpox sera were used in neutralization tests with virus from 4 different zoster vesicle fluids. In contrast to acute chickenpox sera, acute zoster sera taken at comparable times from the onset of the eruption almost completely neutralized zoster virus. All convalescent chickenpox sera neutralized zoster virus, some of them as completely as did convalescent zoster sera.

Great difficulty was encountered in obtaining sufficient infective chickenpox vesicle fluid to test even a small number of sera. However, using the second technique described above, 2 tests were performed, Table IV shows the results obtained with CUN. and CGS. chickenpox fluids.

TABLE IV.—*The Result of Neutralization Tests Using Chickenpox Vesicle Fluids*

Patient	Duration of rash when serum taken (days)	Number of focal lesions in each of two culture tubes: Chickenpox fluid (CUN.)	Chickenpox fluid (CGS.)
		Zoster sera	
C. H—	3	—, —	0, 0
	25	—, —	0, 0
M. M—	5	0, 0	0, 0
	26	0, 0	0, 0
		Chickenpox sera	
P. D—	4	2, 0	0, 1
	25	0, 0	0, 0
F. U—	1	9, 7	10, 9
	25	0, 0	0, 0
Control tubes.	Horse serum only	20, —*	15, 14

— Sera not tested.
—* Non-specific degeneration of amnion cell sheet.

Because of the small number of lesions in the control tubes, these results are less significant than those with zoster vesicle fluid. Even so, it would appear that cross neutralization has occurred.

Tests with suspensions of infected amnion cells as source of virus

Because of the difficulty of obtaining sufficient infective vesicle fluid, from chickenpox cases particularly, a few tests were made using infected amnion cell suspensions.

One test was made by the second method used in the tests with the zoster vesicle fluids except that the concentration of test serum in each tube was 20 per cent. The cell suspension (chickenpox strain CDT.) was obtained by treatment of a single tube culture with 5 ml. of 0·25 per cent. Difco trypsin for 10 min. at 37°. The cells were dispersed as much as possible with a Pasteur pipette and then centrifuged at 2000 r.p.m. for 10 min. The supernatant trypsin was poured off and the cells resuspended in Hanks' solution served as virus inoculum. The sera were those used in the experiment recorded in Table IV. The results are shown in Table V.

TABLE V.—*The Result of a Neutralization Test Using Chickenpox-infected Amnion Cells as Virus Inoculum*

Patient	Duration of rash when serum taken (days)	Number of focal lesions in each of 2 culture tubes: Result on 3rd day	Result on 6th day
		Zoster sera	
C. H—	3	16, 6	33, 22
	25	4, 3	12, 12
M. M—	5	12, 10	44, 50
	26	1, 1	12, 18
		Chickenpox sera	
P. D—	4	12, 23	28, 32
	25	1, 0	5, 5
F. U—	1	—*, —*	—*, —*
	25	13, 3	26, 8

—* Non-specific degeneration of amnion cell sheet.

TABLE VI.—*The Result of a Neutralization Test with Zoster Vesicle Fluid (ZPQ.) and Sera from Individuals with a Past History of Chickenpox*

Patient	Time since chickenpox	Number of focal lesions in culture tubes: Result on 5th day	Result on 10th day
J. R—	40 yrs.	4, 4	8, 10
M. D—	18 ,,	0, 1	0, 1
D. E—	15 ,,	6, 5	8, 7
B. K—	13 ,,	2, 2	3, 2
T. I—	13 ,,	0, 0	0, 0
J. J. B—	17 weeks	0, 0	0, 0
D. T—	11 ,,	0, 2	1, 3
J. J. B—	3 days	31, 37	61, 70
F.M. convalescent zoster serum		0, 0	1, 0

Unfortunately, no horse serum control was set up and the tubes containing acute serum F.U., which might have served as negative controls, showed non-specific degeneration of the amnion cells. Nevertheless, there was a definite suggestion, both in the above test and in another with zoster-infected amnion cells, that chickenpox and zoster convalescent sera, although failing to effect complete neutralization, reduced the number of focal lesions. This result confirms the findings of Weller and Witton (1958).

In some experiments convalescent serum did not markedly reduce the number of focal lesions in comparison with a control acute chickenpox serum. But with the convalescent sera a striking reduction in the size of the lesions was observed. This is illustrated in figures 4, 5 and 6. The size of the lesions produced by zoster virus (strain ZPE., first passage) is inhibited equally by both zoster and chickenpox convalescent sera.

The persistence of neutralizing antibody after chickenpox

Sera from 7 persons with a past history of chickenpox were tested against zoster vesicle fluid. As controls, an acute chickenpox serum and a convalescent zoster serum were included. The acute chickenpox serum had been shown by previous tests to be almost devoid of antibody (JJB., Table III). The results of the test are shown in Table VI. It will be seen that neutralizing antibody persists in the serum for many years after a clinical attack of chickenpox. This contrasts with our finding that with the technique used complement-fixing antibody was not usually detectable in sera taken some months after the illness (Taylor-Robinson and Downie, 1959).

DISCUSSION

The results recorded above have served to confirm the pioneer studies of Weller on the propagation in tissue culture of virus from chickenpox and zoster cases (Weller, 1953; Weller, Witton and Bell, 1958). Although the technique differed somewhat from that of Weller in that stationary cultures of human amnion cells were used for most of the experiments reported here, the cytopathic changes observed were similar. No differences in behaviour were noted between strains derived from zoster cases and those from chickenpox. The same difficulty in obtaining cell-free infective virus from infected cultures was encountered, although on one occasion a small amount of such virus was obtained. Gold and Robbins (1958) also found that, in some experiments, infection of tissue cultures was achieved by means of cell free culture material.

The difficulty of demonstrating virus in the fluid phase of tissue culture is in sharp contrast to the finding with vesicle fluids. In such fluids virus appears to be, for the most part, extracellular; cell-free filtrates of such fluids may, on occasion, have an infective titre almost as high as the unfiltered fluid. The presence of such free virus in vesicle fluid and presumably in the exudate from lesions on mucous membranes probably accounts for the high infectivity of varicella cases.

The absence of demonstrable infective virus in the fluid phase of the cultures presumably accounts for the focal nature of the lesions in tissue culture. However, the fact that immune serum in the medium may limit the number and size of lesions produced by infective cell suspensions suggests that some inhibition of spread of virus from cell to cell is effected by antibody. It would seem that virus

must pass extracellularly from cell to cell for such neutralization to take place. The difficulty of demonstrating virus in the fluid phase of tissue cultures suggests that the amount released into the medium is small or that it is unusually labile and rapidly undergoes inactivation. Electron micrographs of tissue cultures infected with varicella virus show virus particles apparently outside as well as within cells (Tournier *et al.* 1957).

Virus in vesicle fluid, because of its extracellular position, is more effectively neutralized in tissue culture by the immune serum in the medium. In tests using vesicle fluid as inoculum complete neutralization was effected both by convalescent chickenpox and convalescent zoster sera. As in the tests for complement-fixing and precipitating antibodies, there was complete cross reaction between sera from the two clinical conditions against virus from either entity. However, in the neutralization experiments, dilutions of sera were not tested. Consequently it has not been shown that convalescent zoster sera have a higher concentration of neutralizing antibody than convalescent chickenpox sera; such a difference was found in relation to complement-fixing and precipitating antibody. In the experiments of Weller and Witton (1958) and also in this work the antibody content of acute zoster sera was usually greater than that observed in chickenpox sera taken at the same time in the eruptive phase of illness.

The evidence from laboratory studies now strongly supports the epidemiological evidence that chickenpox and zoster are due to the same virus. The results of antibody tests reported in the present series of papers, and particularly the earlier and greater antibody response observed in zoster cases, support the view that zoster is a second clinical manifestation of infection with varicella virus in persons whose immunity from the primary infection, whether clinical or subclinical, has waned.

SUMMARY

Virus from 21 cases of zoster and 22 cases of chickenpox was grown in tissue cultures of human fibroblasts or human amnion cells. In sheets of the latter cells, the lesions produced are more circumscribed and more readily counted and for this reason amnion cell cultures were preferred for neutralization tests.

The lesions produced were focal in character and infection spread by involvement of contiguous cells as described by Weller. The changes produced by virus from chickenpox vesicle fluid were indistinguishable from those produced by virus from zoster. Demonstrable virus remained for the most part within the cells and only occasionally was a small amount of virus found in the culture fluid. In vesicle fluid, virus was mostly extracellular and survived for many months storage at $-70°$. Suspensions of infected tissue culture cells, on the other hand, were not found to be infective after storage at this temperature for more than a few days.

Virus in vesicle fluid was more effectively neutralized in tissue cultures by immune serum than was virus in suspensions of infected tissue culture cells. Paired sera from 7 cases of zoster and paired sera from 9 cases of chickenpox were used in neutralization tests with vesicle fluid virus. Both zoster and chickenpox convalescent sera in the culture medium produced almost complete neutralization of virus from zoster or chickenpox vesicle fluid.

Although the number of focal lesions was not always reduced in neutraliza-

tion tests carried out with infected amnion cell suspensions, there was reduction in the size of the lesions and this was sometimes very striking.

Sera from individuals who had suffered from chickenpox many years before showed significant neutralizing power although they had previously been shown to have no complement-fixing antibody.

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THE RESPONSE OF MICE TO LARGE INTRAVENOUS INJECTIONS OF ECTROMELIA VIRUS

I. THE FATE OF INJECTED VIRUS

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THERE are many viruses, such as smallpox and yellow fever, which are clearly disseminated throughout the body in the course of infection. This dissemination inevitably occurs via the bloodstream. To understand the spread of viruses through the body, it is therefore of importance to know what happens to them when they enter the bloodstream. Infectious ectromelia of mice (mousepox) is a good laboratory model for a generalized infection due to a large virus (Fenner, 1949), and a study of the fate of ectromelia virus injected intravenously into mice was undertaken.

MATERIALS AND METHODS

Virus

The Moscow strain of ectromelia virus was used, in the form of a suspension of infected mouse liver. To produce stock virus preparations, mice were injected intravenously with a large dose of virus, and their livers removed at the time of sickness or death 2 days later. Livers were ground in a chilled mortar and pestle, suspended in about 5 times their volume of gelatine saline (0·5 per cent gelatine in buffered saline) and treated for 3 minutes at maximum intensity with a Mullard 50 watt ultrasonic disintegrator. The suspension was then centrifuged for 10 min. at 1500 *g*, and the supernate stored in aliquots at either —20° or —60°. Ultrasonic treatment was found to increase slightly the yield in the supernate from such liver suspensions ; it had no detectable effect on the titre of thawed stock material.

Stock liver suspensions were reddish-brown in colour, rather viscous, and clearly contained large amounts of non-viral material. Occasionally, purer preparations were made by fluorocarbon extraction of infected livers (Gessler, Bender and Parkinson, 1956). The final extract was concentrated 10 times by centrifuging for 12 min. at 23,000 *g* and suspending the deposit in one tenth the volume of gelatine saline with the help of the ultrasonic disintegrator.

Mice

Outbred multicoloured mice of the Walter and Eliza Hall Institute, Melbourne strain, were used.

Infectivity titrations

Titrations in eggs.—These were carried out by the pock counting method on the chorioallantoic membrane, as described by Burnet and Lush (1936). Five eggs were inoculated with each dilution of material. Ectromelia pocks are rather variable in their appearance. Often they are in the form of small, rounded, easily counted pocks, but sometimes they occur as even smaller and fainter opacities, and sometimes as mere white streaks on the membrane. Occasional membranes have few or no pocks even though they have been inoculated with scores of pock forming units. Nevertheless, satisfactory and reproducible end points were obtained if certain precautions were taken. Firstly, each calculation of titre was weighted

(Burnet and Faris, 1942) by using only the 2 or 3 highest counts. Secondly, no titrations were based on pock counts of less than 5 or more than 50, for if there are very few pocks it is easy to confuse them with non-specific lesions, and if there are very many the titre obtained is lower than when the same material is diluted before inoculation. Thirdly, since concentrated tissue suspensions or blood often produce non-specific lesions, such materials were usually titrated in mice. Finally, whenever there was great variation in the counts from a given titration, or when there were less than 3 membranes available with countable pocks, the titration was repeated.

An estimate of the accuracy and reproducibility of this titration method was obtained by making three $10^{-6.5}$ dilutions of a stock suspension of virus and inoculating each dilution to 5 groups of 5 eggs. Titres, as calculated from 13 groups of eggs with the precautions outlined above, varied from $10^{7.6}$ to $10^{7.9}$. One group of eggs gave a titre of $10^{7.5}$, and another group was discarded because only 2 of the membranes had pocks which could be counted. Differences in titre of more than $10^{0.3}$ are therefore unlikely to be due to titration inaccuracies.

Titrations in mice.—Each tenfold dilution of virus in gelatine saline was injected (0·05 ml.) into 5 mice, either subcutaneously into the footpad, or intraperitoneally. Mice were observed for 14 days, and the survivors then killed and infection scored by swollen foot-pads (more common in survivors), liver or spleen lesions, and swollen iliac lymph glands. In many titrations of this sort, it was found that the ID_{50} titre (as calculated by the method of Reed and Muench (1938)) was up to 10 times as high as the pock forming unit (pfu) titre.

Haemagglutinin titrations

Twofold dilutions (0·25 ml. volumes in perspex trays) were made in normal saline containing 1 per cent normal rabbit serum to inhibit non-specific lipid haemagglutination, and one drop of a 2 per cent suspension of washed red blood cells from a susceptible fowl was added to each cup. After incubation for 30 min. at 36°, a shake, and reincubation for one hour, the end points, based on the pattern of agglutinated cells, were recorded.

Complement fixing antigen titrations

Serial twofold dilutions (0·25 ml.) of material were made in CaMg saline in perspex trays. One drop of a 5 per cent solution of inactivated hyperimmune mouse serum was added to each dilution, followed by 2–3 MHU of complement. Trays were held at 4° overnight before adding a drop of 2 per cent sensitized sheep cells. End points (50 per cent haemolysis) were read after incubation at 36° for 30 min., a reshake, and a further 3 hr. at room temperature. Infected livers were found to fix small amounts of complement in the absence of immune serum, but none of the many normal livers tested did so.

Fluorescence microscopy

Liver samples were frozen in containers in a dry ice-alcohol bath, and stored at −20°. Sections (6μ) were cut with a Cambridge Rocker Microtome in a standard −20° refrigerator, as described by Louis (1957). They were taken onto dry slides, dried for at least an hour with a fan at room temperature, fixed for 10 min. in acetone and redried for at least 10 min. The ectromelia material was then stained by the indirect method. This was found to be more sensitive than the direct method, even when conjugates for the direct method were prepared without the use of acetone and dioxane (Goldman and Carver, 1957) or with fluorescein isothiocyanate (supplied by the Sylvana Chemical Co., Orange, New Jersey, U.S.A.). A vaccinial-hyperimmune serum was produced in rabbits, and adsorbed thrice with mouse liver powder before use. Sections were treated with this serum for 45 min., washed for 10 min. in 2 changes of buffered saline, and then stained for another 45 min. with a commercially produced fluorescein-labelled sheep anti-rabbit-γ-globulin (Sylvana Chemical Co.) which had also been adsorbed thrice with mouse liver powder. After a final 10 min. wash in 2 changes of buffered saline, sections were mounted in neutral glycerol and observed with a Leitz carbon arc fluorescence microscope. Sections of uninfected tissue were dealt with in the same way to confirm the specificity of staining. Photomicrographs were taken on 9 × 12 cm. plates of HP3 Ilford film.

Sections which had been examined by fluorescence microscopy were stained with haematoxylin and eosin after floating off the cover slips in formol saline. Although staining was often satisfactory, results were sometimes very poor. This was so in spite of numerous modifi-

cations in technique, and it was concluded that there is something unpredictable in the response of very thin frozen sections to fixation and staining.

EXPERIMENTS

Clearance of virus from the blood

A massive intravenous injection of $10^{8.4}$ pfu (about $10^{9.4}$ ID_{50}) of stock virus was given to 6 mice, and pooled tail bleeds were taken hourly and titrated *in ovo*. (Fig. 1). One hour after the injection nearly all virus had disappeared from the blood. There was a further very slight fall in blood titre during the next few hours, after which the titre was almost unchanged until about the thirteenth hour (Mims, 1959). In subsequent experiments it was shown that more than 90 per cent of the

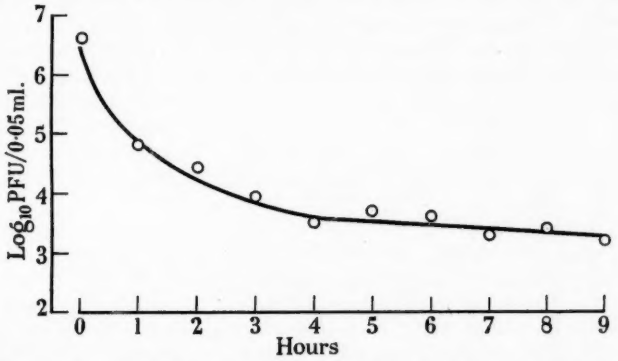

FIG. 1—Clearance of intravenously injected virus from the blood of mice.

injected virus had been removed from the blood within 2–3 min. of injection. Similar clearance rates were obtained when fluorocarbon extracted virus was used.

Adsorption sites

When mice were given a massive intravenous injection of virus, all the injected virus was recoverable in a suspension of the whole mouse made 5 minutes later. In this experiment the skin and gut were removed and the carcass then suspended in 350 ml. gelatine saline in a Waring type blender in the cold. The suspension was given ultrasonic treatment, lightly centrifuged, and then titrated.

It was calculated in other experiments that 5 minutes after such massive intravenous inocula more than 95 per cent of the injected virus was present in the liver. Relatively small amounts were present in other organs, and little or none was present in the gut, for instance. The table shows a typical result.

Virus taken up by cells in the liver within minutes of its injection might at this time be on the surface of cells rather than in them. An attempt was made to wash adsorbed virus out of the liver by severing the great vessels in the thorax and then perfusing 10 ml. of saline under high pressure into the portal vein as early as $1\frac{1}{2}$ min. after the virus injection. The titre of the blanched liver thus

TABLE.—*Location of Virus in the Blood and Organs of a Mouse 5 min. after a Large Intravenous Injection*

Organs	Log_{10} pfu/per organ	Total virus (per cent)
Liver	7·0	95·3
Spleen	5·6	3·8
Lungs	4·6	0·4
Kidneys	4·2	0·1
Gut	<3·0	—
Total blood content	4·5	0·3

obtained was not detectably different from that of a small portion removed before the perfusion. It was concluded that within 1–2 min. of injection most of the virus is either firmly attached to or even inside cells in the liver.

In other experiments intravenously injected Indian Ink was clearly present inside the Kupffer and endothelial cells of livers put into fixative 30 sec. after the

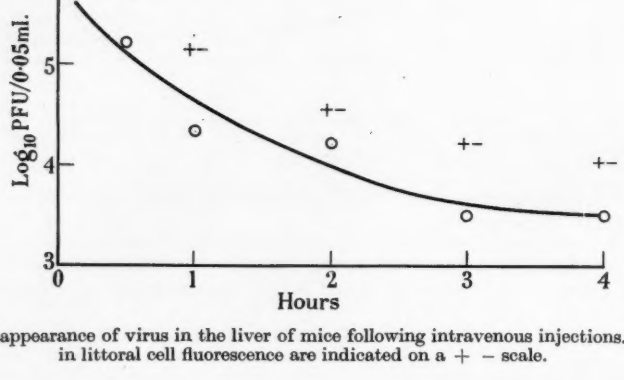

FIG. 2.—Disappearance of virus in the liver of mice following intravenous injections. Changes in littoral cell fluorescence are indicated on a + − scale.

injection (Fig. 5). Again, in the fluorescence microscopy investigations described below, virus was observed in rather than on cells 2 min. after intravenous injections.

Disappearance of virus in the liver

A massive intravenous injection of stock virus was given to many mice, and at intervals 3 mice were killed with ether, portions of their livers removed, pooled, suspended in gelatine saline, and titrated *in ovo*. Complement fixing antigen titrations were also made. Nearly all the virus which had been present in the liver at 5 min. was no longer detectable in infectivity titrations after 1 hr. (Fig. 2) and there was a further slight fall in titre during the next 2 hr.

The CF viral antigen was also taken up by the liver, but it did not disappear, and it was calculated that virtually all the CF antigen injected (10,000 units

was demonstrable in the liver throughout the experiment. This difference in behaviour suggests that most of the CF activity of ectromelia virus preparations is distinct from the infective particles.

Fluorescence microscopy

In a number of different experiments about 10^9 ID_{50} were injected intravenously into mice, and liver samples removed and frozen in a dry ice-alcohol mixture after various time intervals, as follows:

2–5 min. (Figs. 6–7). Littoral cells lining the sinusoids brightly fluorescent. (The cells lining the liver sinusoids include Kupffer cells, endothelial cells, and leucocytes. There is no evidence that they behaved differently in the experiments described here, and since in addition it is thought that they constitute a changeable rather than a fixed population, they are referred to as "littoral" cells). Fluorescent material clearly in rather than on the littoral cells. In general fewer fluorescent cells round central veins. No fluorescence in hepatic cells. 250–300 fluorescent foci per standard field ($10^{5.5}$ μ^2) of which at least 100 included cell nuclei. About 250 littoral cells in haematoxylin and eosin stained sections of normal liver, so that probably all or nearly all of these cells were fluorescent.

30 min. About half as many fluorescent foci visible and these foci considerably less bright.

1 hr. Further fading in brightness of foci.

2 hr. Little change. About 100 fluorescent foci per standard field.

3 hr. (Fig. 8). A few fluorescent granules seen for the first time in littoral cells, otherwise little change. Still no fluorescent hepatic cells.

Lung and kidney were also examined 30 min. after similar massive intravenous injections of virus. Antigen-containing cells (probably macrophages) were seen in the lung, and antigen was present in some of the kidney glomeruli.

Thus virtually all intravenously injected virus is taken up immediately by littoral cells in the liver, and quite probably these cells remove nearly all the virus from the blood in a single passage through the liver. The disappearance of infective virus from the liver during the first hr. is paralleled by a fading and disappearance of specific fluorescence (Fig. 2).

Adsorption sites for smaller viruses injected intravenously

It is probable that when Rift Valley Fever virus is injected intravenously into mice it is present in hepatic cells an hour later (Mims, 1957). As early as 6 hr. after large injections there has been a cycle of growth in hepatic cells and gross histological changes are established. Thus there are marked differences in the fate in the liver of intravenously injected Rift Valley Fever and ectromelia viruses. It seemed possible that Rift Valley Fever virus, on account of its small size, is able to enter hepatic cells directly from the blood, possibly through minute discontinuities in the endothelial lining of sinusoids. Various viruses smaller than ectromelia were therefore injected intravenously into mice, and livers then removed, frozen, sectioned and stained with fluorescein labelled conjugates by the direct method.

Influenza Virus (Strain MEL).—Livers were stained with fluorescein labelled fowl antiserum. Five minutes after the intravenous injection of $10^{9.0}$ ID_{50} of MEL virus (red cell eluate), considerable amounts of antigen were present in

large numbers of littoral cells in the liver. There were no fluorescent hepatic cells. By one hour there had been a marked decrease in the number of fluorescent littoral cells. There was no change in appearances at 3 hr.

Murray valley encephalitis virus.—Livers were stained with fluorescein labelled monkey antiserum (serum kindly supplied by Dr. S. G. Anderson). 10^7 adult mouse IC LD_{50} of a mouse brain suspension were injected intravenously into mice. Virus was present in littoral cells, but not hepatic cells, within 10 min., and there was no change by 2 hr.

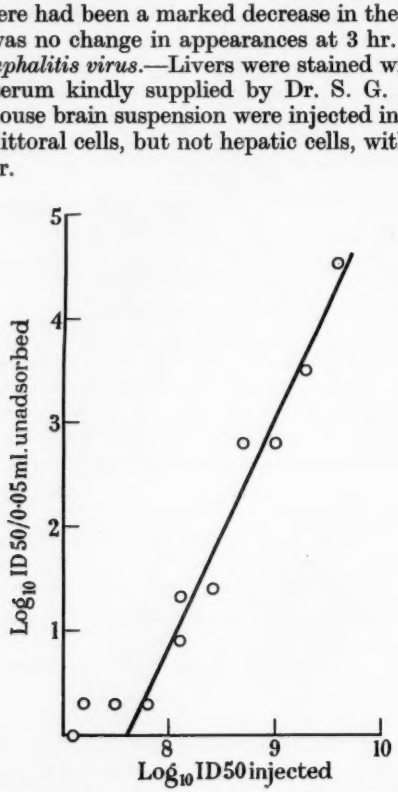

FIG. 3.—Blood virus titres 5 hr. after various sized intravenous injections of virus.

Poliovirus (Type 1).—Livers were stained with fluorescein labelled monkey antiserum. Thirty minutes after the intravenous injection of $10^{8.0}$ pfu of purified Type I virus there were a few fluorescent littoral cells, and these cells were common after one hour. There was no fluorescence in hepatic cells.

It is concluded that all the viruses tested are taken up from the blood by littoral cells in the liver, and an hour or two later have still not reached hepatic cells in appreciable amounts. Uptake is probably slower in the case of poliovirus.

Incomplete clearance of virus from the blood

In experiments where the blood from single mice or pairs of mice was titrated 3–5 hr. after the intravenous injection of virus there was some variation in the amount remaining in the blood after a given injection. Nevertheless, it was clear that increasing, although still small, amounts of virus remained in the blood when larger doses were given. Fig. 3 includes the results from 2 experiments

in which each titration (mouse ID_{50}) was done on pooled blood from 2 mice 5 hr. after the intravenous injection of different doses of virus. As a rule there was no detectable virus left in the blood when less than $10^{6.0}$ ID_{50} were injected.

The amount of virus left in the blood was never more than a small fraction of the amount injected, but the littoral cells in the liver sinusoids take up virus with high efficiency and there seemed no reason why any virus at all should stay in the blood. In a number of experiments it was found that much of the virus left in the blood was cell-associated and was deposited when citrated or heparinised blood was lightly centrifuged. When the virus contained in such blood was injected into other mice most of it stayed in the circulation and, while mice varied slightly in their response, there was often no detectable clearance from the blood within 3 hr. It was concluded that virus which is not cleared from the blood is associated with cells which remain in the bloodstream for at least 3 hr.

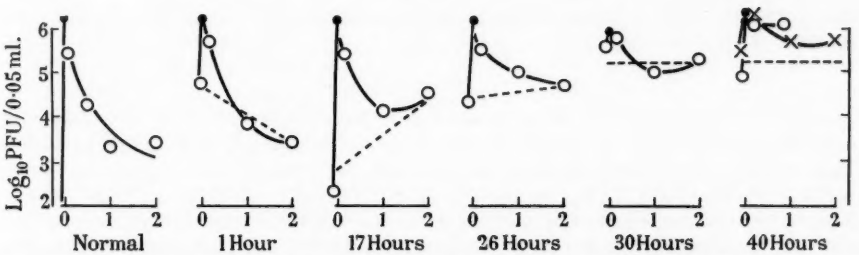

FIG. 4.—Clearance of intravenous challenge doses of virus from the blood of infected mice. The time of challenge is shown in each case, and subsequent hours are indicated on abscissae. Dots on curves show the theoretical immediate titres following the challenge inocula. One of the mice challenged at 40 hr. died an hour later.

In an attempt to identify the blood cells that adsorbed virus, citrated blood was obtained from mice 3 hr. after massive intravenous injections of virus. The cells were sedimented by centrifugation at 550 *g* for 10 min., and it was found that virtually all the sedimented virus was in the buffy coat ; there was little or none in the erythrocyte layer. However, when the supernatant liquid was centrifuged at 2000 *g* for 30 min., which does not bring down virus particles, more than 90 per cent of the virus was sedimented, and smears of the sediment were composed of large numbers of platelets. There was as much virus in this platelet sediment as in the buffy coat. Blood smears from the same mice were examined by fluorescence microscopy, but antigen could not be seen ; probably the amount of virus adsorbed per susceptible cell or platelet was inadequate to give detectable staining.

Clearance of virus from the blood during infection

After massive intravenous doses of ectromelia there is growth of virus in some of the littoral cells lining the sinusoids and in the hepatic cells themselves (Mims, 1959). It was thought possible that this would result in a defective clearance of virus from the blood. A massive intravenous injection of virus was therefore given to many mice, and at different times afterwards a similar injection was given to one of these mice and serial tail bleeds from this mouse were then titrated. It was found (Fig. 4) that the second large dose was rapidly cleared and the blood

titre reduced to the level which prevailed at the particular stage of infection involved. Very late in infection, however, clearance of virus from the blood was impaired. There was a possibility that organs other than the liver adsorb virus late in the infection. To test this possibility, mice in this stage of infection were given a large intravenous challenge dose of rabbitpox virus. Bloods and livers were then titrated for rabbitpox on the chorioallantoic membrane, and the readily identifiable pocks counted at 2 days. It was found that the rabbitpox challenge was not cleared as completely as in normal mice, but that much of it was detectable in the liver after adsorption. It was also found that Indian Ink, when injected intravenously to mice late in infection, was taken up by littoral cells, many of which appear histologically normal at this time.

There is thus no detectable defect in the blood clearing mechanism until a late stage in infection. It is possible that the defective clearing late in infection is a vascular phenomenon, because it was clear from histological studies that blood was circulating through only a limited number of liver lobules at this time.

DISCUSSION

Intravenously injected virus particles are probably dealt with in the same way as inert particles such as colloidal chromium phosphate (Dobson, 1957) or Indian Ink. The rate of clearance depends on particle size. Dobson, Gofman, Jones, Kelly and Walker (1949) reported that the half life of large particle colloids injected intravenously into mice was 30–60 sec. whereas for small particle colloids the half life was 30–80 min. The clearance rate of virus particles may also depend on size, for the half life of ectromelia is less than 3 min. while the half life of the much smaller Rift Valley Fever virus is about 20 min. (Mims, 1956).

Large particles are present inside phagocytic cells within a very short time of injection. Parks and Chiquoine (1957) showed by electron microscopy that intravenously injected colloidal gold particles were present on the surface of littoral cells in the mouse liver 15 sec. after injection, and that phagocytosis was occurring within 2 min. Indian Ink is also phagocytosed by the same cells within a minute of injection (Fig. 5). Virus particles are probably taken up by phagocytosis in the same way, and ectromelia is inside littoral cells within 2 min. of injection.

Dobson and Jones (1952) and Dobson *et al.* (1949) showed that although the liver was largely responsible for the rapid clearance of large particles, the relative importance of other organs depended on the species of animal. In the mouse about 95 per cent of such particles were taken up by the liver, 1–4 per cent by the spleen, and relatively little by other organs. In the rabbit, however, the liver was less active (85 per cent) and the bone marrow more active. Ectromelia virus, apparently, (Table I) is taken up by the organs of the mouse in much the same proportions as are inert particles.

Whether or not the endothelial cell lining of sinusoids is morphologically continuous, the phagocytic mechanism is so efficient that however much virus is injected, it does not reach hepatic cells. It was shown by Erickson, Hensley, Fields and Libby (1957) that when ^{32}P labelled TMV was injected intravenously to mice, it was present in Kupffer cells but not hepatic cells an hour later. Because of the very rapid uptake by littoral cells in the liver, neither the phagocytic cells in other organs nor the cells in the blood have a chance to take up more than a

small fraction of the amount injected. Even littoral cells near the central veins, which first encounter the blood as it leaves the sinusoids, have less opportunity to take up virus, and become less fluorescent than other littoral cells.

Each of the 4 smaller viruses tested was taken up by littoral cells in the liver after intravenous injection, and none could be detected in the hepatic cells within the next few hours. Viruses present in the blood sometimes grow in hepatic cells, and there are three ways in which they could reach these cells. Firstly, they might pass through discontinuities in the littoral cell lining of sinusoids and thus reach the hepatic cells directly. This does not occur to a detectable extent in the case of the four viruses tested here, in spite of the fact that poliovirus, one of the smallest viruses, was used. Secondly, viruses may reach hepatic cells by being taken up by littoral cells and then passively passed through to hepatic cells, in the way that has been described for fine fat droplets by Jaffé and Berman (1928). This may be the way in which Rift Valley Fever virus reaches hepatic cells within an hour of intravenous injection. Thirdly, viruses may reach hepatic cells by "growing through" littoral cells, as is described for ectromelia virus in the next paper (Mims, 1959).

The specific fluorescence of littoral cells fades during the first hour after the injection and this, when correlated with the considerable decrease in liver titre which occurs at the same time, is evidence of an eclipse phase. Moreover, the fading of fluorescence suggests that the littoral cells digest or degrade the protein coat of the virus which is responsible for fluorescent staining. It seems probable that these reticulo-endothelial cells start digesting the virus particle in the same way that they would start digesting any other phagocytosed particle.

Virus which is not cleared from the blood is not cleared because it is present on or in leucocytes and platelets. The blood cells do not take up a constant proportion of the injected virus; if this were so, the slope of the curve in Fig. 3 would be 45°. The presence of cell-associated blood virus accounts for the levelling-out of adsorption curves. The same phenomenon has been observed when rabbits are injected intravenously with staphylococci (Rogers, 1956).

There is unimpaired clearance of intravenously injected virus until a later stage of infection, and it is probable that intact littoral cells in the liver are responsible for most of this clearance. Again, Rogers (1956) found an unimpaired clearance of staphylococci in infected rabbits. Clearly, while there is unimpaired clearance of virus from the blood, there are only two ways in which a viraemia can be maintained; either by the release of virus into the blood as fast as it is cleared, or alternatively, by virtue of the fact that virus in the blood is associated with cells so that it cannot be cleared in the normal way. This second mechanism operates for the viraemia which follows large intravenous doses of ectromelia virus.

SUMMARY

Ectromelia virus is virtually all cleared from the blood of mice within a few minutes of a massive intravenous injection, and is taken up by the littoral cells lining the liver sinusoids.

However much virus is injected, none reaches hepatic cells.

During the first hour after injection there is a great decrease in the amount of demonstrable antigen in littoral cells, as shown by fluorescence microscopy, and in the amount of infective virus.

It is thought that intravenously injected virus particles are dealt with in the same way as intravenously injected inert particles.

A small proportion of the injected virus becomes associated with circulating platelets and perhaps leucocytes, so that it cannot be removed from the blood by littoral liver cells.

There is unimpaired clearance of intravenously injected virus until a late stage in infection.

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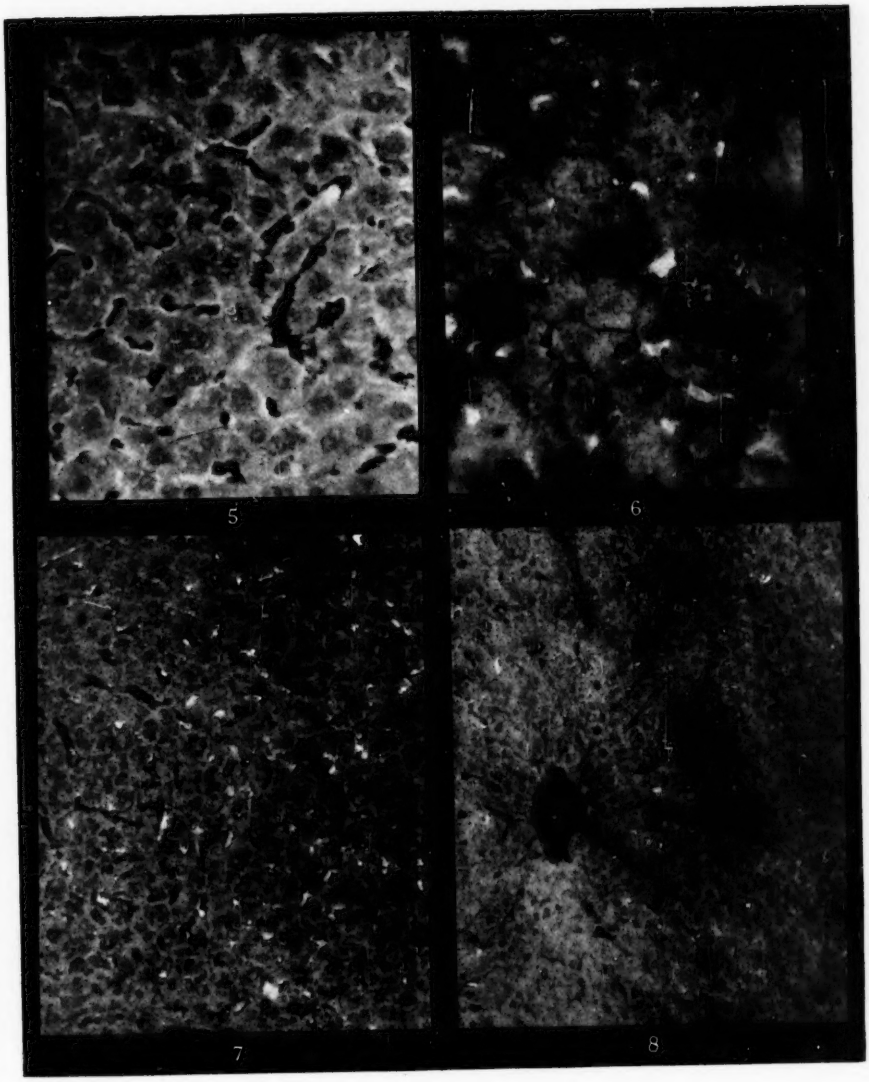
EXPLANATION OF PLATES

FIG. 5.—Mouse liver 30 sec. after intravenous injection of Indian Ink. Haematoxylin and eosin. × 500.

FIG. 6.—Mouse liver 2 min. after massive intravenous injection of ectromelia virus. Fluorescent antibody stain. Antigen present in many littoral cells. × 500.

FIG. 7.—Mouse liver 5 min. after massive intravenous injection of extromelia virus. Fluorescent antibody stain. × 150.

FIG. 8.—Mouse liver 3 hr. after massive intravenous injection of ectromelia virus. Fluorescent antibody stain. Marked fading and disappearance of fluorescence. × 150.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THE RESPONSE OF MICE TO LARGE INTRAVENOUS INJECTIONS OF ECTROMELIA VIRUS

II. THE GROWTH OF VIRUS IN THE LIVER

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It was shown in the previous paper that when ectromelia virus is injected intravenously into mice it is taken up by the littoral cells lining the liver sinusoids. However much is injected, it does not reach hepatic cells. Nossal and de Burgh (1954) investigated the growth of ectromelia in the liver of mice following massive intravenous injections of virus. They decided that there was a stepwise increase in virus titre in the infected liver, occurring over the course of at least 24 hr. From the histological appearances 18 hr. after the injection each hepatic cell was judged to have been infected from the start, and they therefore suggested that there was a stepwise increase in titre in individual hepatic cells. If, however, injected virus does not infect hepatic cells at all, Nossal and de Burgh's conclusions are almost certainly incorrect and the growth and spread of virus in the liver deserves a fuller investigation. A detailed study of the matter has therefore been made, using the fluorescent antibody technique in addition to infectivity titrations.

MATERIALS AND METHODS

These were identical with those already described (Mims, 1959).

EXPERIMENTAL

Preliminary

Even with the largest intravenous inocula that could be given ($10^{8.5}$ pfu), mice did not begin to sicken and die until at least 24–26 hr. afterwards.

At the time of death (Table I) there were very large amounts of virus in the liver, smaller amounts in the spleen, and very little elsewhere. The virus content of some organs is attributable to the blood in them. Only a very small proportion of the virus produced in the liver appears in the blood, and nearly all of this circulating virus was found to be cell-associated, and could not be removed by 4 washes in saline. Although virus from the liver might have been expected to drain into the biliary passages and into hepatic lymph, both the bile and the hepatic lymph gland in the porta hepatis contained little virus.

Ectromelia will grow in a wide variety of organs and tissues of mice, but 99 per cent of the virus in a large intravenous inoculum is very rapidly taken up by the liver. Virus which is not taken up from the blood is mostly cell-associated (Mims, 1959) so that there is little opportunity for any but small amounts to reach other organs and tissues. There is, however, growth in other organs and

TABLE I.—*Organ Titres at the Time of Sickness or Death on the Second Day After a Massive Intravenous Injection of Virus*

Material	Log_{10} pfu/0·05 ml. tissue
Liver	8·1
Spleen	7·6
Lung	6·5
Kidney	5·2
Blood	5·0
Sternum	4·9
Bile	4·8
Pancreas	4·6
Heart	4·6
Testicle	4·4
Lymph glands	up to 4·0
Brain	3·8
Hepatic lymph gland	3·4
Skin	3·0
Conjunctival washings	0
Naso-oral washings	0

tissues if virus is injected extravascularly. When virus was injected intracerebrally or into the foot-pad, for instance, the brain and popliteal lymph gland titres on the second day were 100 times as high as after intravenous injections.

There is a coagulation defect in ectromelia-sick mice. Coagulation times of more than 30 min. (capillary tube method) were frequently encountered. This defect is perhaps attributable to a prothrombin deficiency which may occur in mice a short time after extensive liver damage (Mims, 1956).

Growth curves

Many mice were given an intravenous injection of $10^{8.2}$ pfu of virus contained in 0·1 ml. of liver suspension, and at hourly intervals 3 mice were killed with ether and their bloods and livers removed and pooled. Infectivity titrations of bloods and livers were made *in ovo* and complement fixing antigen and haemagglutinin titrations of the liver samples were also made. The results in Fig. 1 show that there is a stepwise increase in both blood and liver titres. The liver curves were confirmed in another experiment, and the blood curves in several experiments in which serial tail bleeds were made from the same mice throughout the experiment.

After adsorption is completed at 1–2 hr. (Mims, 1959) there is a period of 2–3 hr. during which titres remain fairly steady. At 6–7 hr. there is a sudden increase in liver titre which is not reflected in the blood titre. From the 10th to the 12th hr. the liver titre is steady again, and at 13 hr. there is another increase which is now accompanied by an increase in blood titre. There are further stepwise increases in liver titre at 18, 24, and 28–30 hr. Liver CF antigen titres follow infectivity titres closely, and blood titres follow liver titres fairly closely. There is thus a stepwise increase in liver and blood titres following massive intravenous inocula. Sickness was first detectable in some mice by 26 hr. and the first deaths occurred at 27 hr. At 33 hr. only 3 of the remaining 16 mice were alive, and these 3 sick mice were killed as the sample.

Stock liver suspensions of virus have HA titres of up to 320, and the HA titration results were therefore unexpected, for all livers had titres of less than 10, except for the 30 hr. sample, which contained 640 HA units/0·25 ml. liver suspension. Another experiment was carried out, in which the livers from mice

sick or dead 2 days after a massive intravenous dose of virus were separately suspended and titrated for HA. Most livers contained little or no HA, while some contained large amounts (Table II). It is concluded that the HA titres of pooled liver suspensions are caused by the occasional livers with high titres; most do not produce much haemagglutinin. There was, moreover, little or no detectable haemagglutinin in any of the separately titrated livers from 9 mice sick or dead 8–10 days after a small foot-pad injection of virus.

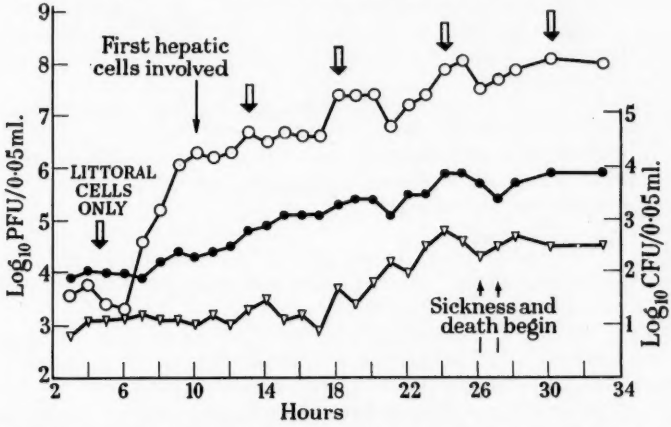

FIG. 1.—Growth curves for liver and blood virus after a massive intravenous injection of virus.

○——○ liver infectivity titres
●- - -● liver CF antigen titres
▽——▽ blood infectivity titres.

Principal fluorescence findings indicated above curves and marked increases in fluorescence shown by thick arrows.

TABLE II.—*Haemagglutinin Titres Attained in Individual Livers After Massive Intravenous Injections of Virus*

Liver Haemagglutinin Titre	Number of Mice
640	2
160	1
40	3
10	1
<10	5

Fluorescence microscopy

At least 2 of the 3 livers in each hourly sample from the growth curve experiment were examined as described (Mims, 1959).

3 hr.—Small number of moderately fluorescent littoral cells.

4–5 hr.—Marked increase in number and brightness of fluorescent littoral cells. A few fluorescent mononuclear cells, probably leucocytes, seen in sinusoids.

6–9 hr.—Increase in brightness continues. By 9 hr. 70–100 fluorescent cells per standard field ($10^{5.5}$ μ^2), and some of these seem to be leucocytes. No fluorescence in hepatic cells.

10 hr.—A hazy fluorescence has appeared round some of the fluorescent littoral cells, involving up to 5 or 6, but more commonly 1–2 neighbouring hepatic cells.

11–12 hr.—A faint uniform fluorescent haze now fills and outlines the involved hepatic cells, and some of them contain a few bright fluorescent granules. About 50 fluorescent hepatic cell foci per standard field.

13 hr.—Marked increase in extent and brightness of fluorescence. A larger proportion of the hepatic cell foci consist of 3–4 rather than 1–2 cells. Many more hepatic cells contain bright fluorescent granules.

14–17 hr.—Slight increase in brightness. Up to 60 fluorescent hepatic cell foci per standard field, together with 5–10 cells in sinusoids with no neighbouring fluorescent hepatic cells.

18 hr.—Foci markedly brighter and larger, and beginning to merge and become uncountable. Non-fluorescent areas still clearly marked.

19–23 hr.—Slight increase in brightness but no increase in size of foci.

24 hr.—Marked increase in extent of fluorescence. Non-fluorescent areas still occur, mostly round central veins.

25–28 hr.—No change.

30–33 hr.—Further increase in extent of fluorescence. Almost the entire section is brightly fluorescent, and there are now only occasional non-fluorescent hepatic cells round some of the central veins.

There were thus 5 distinct increases in fluorescence, and these correspond fairly closely with the stepwise increases in titres (Fig. 1). Most of the fluorescent littoral cells seen at 9 hr. gave rise to fluorescent hepatic cell foci which then enlarged at each increase in fluorescence. It was not possible to decide whether an increase in the number of fluorescent foci occurred after 17 hr.

At no time were fluorescent hepatic cell nuclei seen.

There were differences in the appearance of livers of different mice at given times. For instance, only one of the three 24 hr. livers showed a distinct increase in fluorescence as compared with earlier livers, and the development of fluorescent hepatic cell foci between 11 and 17 hr. varied. In each case the liver showing the most advanced change is described because it is clearly such a liver which determined the titre of the sample.

In the early stages of this work it was often difficult to decide whether or not any hepatic cells were fluorescent before 10 hr., and although the fluorescence and the haematoxylin and eosin pictures taken of identical fields never showed hepatic cell fluorescence, this was a laborious way of excluding such a possibility. A preliminary experiment was therefore done in which mice were given a large intravenous injection of Indian Ink in saline, and at intervals afterwards their livers were fixed in formol saline and stained with haematoxylin and eosin. Within one minute of the injection most of the littoral cells were very clearly outlined by the Indian Ink present in them (Mims, 1959). By 1 hr. the Indian Ink in cells had been concentrated into larger lumps and there was an increase in the number of Indian Ink containing cells in the sinusoids. Even by 24 hr., however, there was no Indian Ink present in hepatic cells. Thus Indian Ink could be used to label littoral cells in sections. The same amount was therefore given intravenously to another group of mice, followed 30 min. later by a massive intravenous injection of ectromelia virus. Livers were examined by fluorescence microscopy during the first 9 hr., and in this experiment a Zeiss standard microscope was fitted with a mercury vapour lamp ultraviolet source as well as phase contrast equipment.

A given field of an unfixed section could then be examined by fluorescence microscopy and immediately afterwards by phase contrast. Littoral cells were readily identified under phase contrast by the Indian Ink contained in them, and in the course of many observations fluorescence was only seen in cells which contained Indian Ink. It was concluded that virus antigen was not present in hepatic cells during the first 9 hr. after massive intravenous injections of virus. This conclusion was subsequently confirmed on many occasions as experience was gained in distinguishing hepatic from littoral cells in fluorescent preparations.

Although there is probably a cycle of growth in littoral cells, it was thought possible that the increase in littoral cell fluorescence and in liver titre which began at 6–7 hr. might have been a result of the liberation of virus into the blood from other organs or tissues and its immediate uptake by littoral cells An experiment

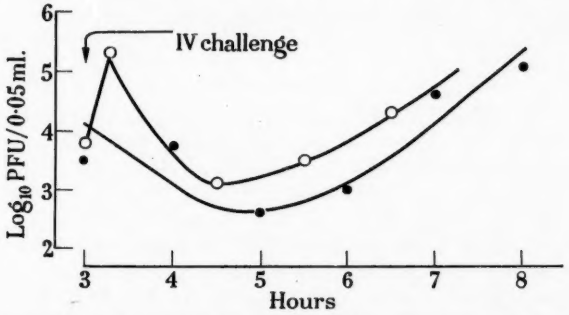

FIG. 2.—Disappearance of a second intravenous dose of virus in the liver of mice 3 hr. after a preliminary intravenous dose.

○———○ liver titres in mice reinoculated at 3 hr.
●———● liver titres in control mice.

was therefore done in which mice were given a large intravenous dose of virus, followed 3 hr. later by another similar dose. Pooled liver titres were obtained in the usual way. The results (Fig. 2) show that when the second dose of virus was introduced into the bloodstream the liver took it up (see also Mims, 1959) but that $1\frac{1}{2}$ hr. later the liver titre was the same as if no extra dose had been given. An increase in liver titre occurred at the usual time, 6–7 hr. after the first injection. It is concluded that this increase in liver titre is the result of a cycle of growth in littoral cells.

Routine microscopy

Livers were also fixed in formol saline and stained with haematoxylin and eosin. There was a distinct increase in the number of cells in the sinusoids which was well marked by 9 hr. The extra cells were mononuclear and included medium and small lymphocytes. Some of the littoral cells were pyknotic by 14 hr., but there were no obvious histological changes in hepatic cells until 17–18 hr., when small necrotic foci were visible. These foci became larger and more common in later samples. In such foci the hepatic cell nuclei became swollen and the cytoplasm foamy ; the nuclei then either began to disintegrate into small pyknotic fragments

or disappeared from the cell, while the cytoplasm often took on a homogeneous eosinophilic appearance.

In some of the 23 hr. and later livers, swollen hepatic cell nuclei, often with fragments of attached cytoplasm, were seen lying apparently free in sinusoids or small veins. A search was therefore made for them in Leishman stained peripheral blood smears taken from sick mice. A number of pyknotic nuclear fragments, often accompanied by a fringe of cytoplasm, were present in these smears. It was difficult to say whether or not they were hepatic cell fragments. Some of them looked like abnormal platelets, and others like fragmented lymphocytes. Lymphocytes with pyknotic and partially fragmented nuclei were also present. Blood from sick mice contained up to 5 times the normal number of leucocytes, although there was no marked change in the differential count. Blood from mice which were not yet sick, 24 hr. after massive intravenous inocula, had normal cell counts and contained no detectable cell fragments.

Discussion

In the previous paper it was shown that when mice were given large intravenous injections of ectromelia virus there was immediate uptake of the greater part of the inoculum by the littoral cells lining the liver sinusoids. However large the dose injected, virus could not be detected in hepatic cells. It has been shown in the experiments reported above that the first hepatic cells are not infected until virus has been released following a cycle of growth in the littoral cells. Subsequently there is spread from infected to neighbouring uninfected hepatic cells at the end of each growth cycle. Both the foci of infection, and the amount of virus in the liver, enlarge in a stepwise fashion. The initial growth cycle in littoral cells lasts 9 hr. (time at which 50 per cent of the virus is produced), and there are then 3 or 4 cycles of growth in hepatic cells, each lasting about 5 hr. (period between 50 per cent production time of successive cycles).

After the second hepatic cell cycle at 18 hr., foci are becoming confluent, so that a count of infected foci is no longer possible. It is therefore uncertain whether the foci increase in number late in infection or whether they increase only in size. Sections gave a general impression of a moderate increase in the number of foci. At the time of sickness and death, however, there are few uninfected liver cells. In some livers the initial spread of infection from littoral to hepatic cells was on a less extensive scale, and it was probably in such livers that a fourth hepatic cell growth cycle occurred before death, which was therefore delayed a few hr.

Virus is present in nearly all littoral cells within a short time of the injection. Since only 70–100 of the initial 250 fluorescent cells per " standard field " were fluorescent at 9 hr., it is concluded that about one in three of these cells support virus growth. There were about 60 fluorescent hepatic cell foci at 11–17 hr., and this means that nearly all the littoral cells which produce virus initiate foci by infecting adjacent hepatic cells.

In the 13 hr. liver sections there were commonly 3–4 adjacent fluorescent hepatic cells, and in 3 dimensions such foci might contain a larger number of cells. This was during the first cycle of growth in hepatic cells and it might seem unexpected that virus should reach a number of adjacent hepatic cells from a single infected littoral cell. It is probable, however, that each littoral cell is in

direct contact with at least 2 hepatic cells, because there are only about half as many littoral cells as hepatic cells. There is, moreover, a fluid filled space visible by electron microscopy between littoral and hepatic cells (Parks, 1957), and virus liberated into this space from a single littoral cell might well infect a number of neighbouring hepatic cells.

In the previous paper it was noted that immediately after large intravenous injections of virus, there tended to be fewer fluorescent littoral cells in the vicinity of the central veins. This may be why the hepatic cells in this region tend to be the last to be infected.

Both in the littoral cell and in the first hepatic cell growth cycles there was an increase in fluorescence about 2 hr. before a rise in infectivity or complement fixing antigen titre was demonstrable. This could be because the protein antigen seen by fluorescence microscopy is manufactured before the virus becomes infective or fixes complement.

There were no detectable histological changes in hepatic cells until 17–18 hr. (Nossal and de Burgh, 1954) and this was at the end of a first cycle of growth in some of these cells. Pyknotic littoral cells, however, were present by 14 hr.

In spite of the sudden increase in liver titre during the littoral cell growth cycle at 6–10 hr., there was no detectable increase in blood titre at this time, the first distinct increase occurring in association with the first hepatic cell growth cycle. Since most of the circulating virus is associated with leucocytes and platelets, an increase in blood titre might involve an increase in the number of circulating infected leucocytes and platelets. This increase may not occur until after the littoral cell growth cycle. There was an accumulation of leucocytes in the liver sinusoids by 9 hr., and it is possible that some of these then became infected as a result of their proximity to infected littoral cells, and were subsequently released into the blood stream. Additional infected leucocytes and platelets, however, may have come from the bone marrow, where a limited growth of virus occurs (Table I).

Degenerate cell fragments appear in the peripheral circulation at the time of sickness. This also occurs in the more rapidly lethal infection of mice with Rift Valley Fever Virus (Mims, 1957). Nothing is known of the possible role of this phenomenon in causing sickness and death.

When 10^4 pfu of virus were injected intravenously to mice, small numbers of fluorescent littoral cells were seen at 11 hr. (unpublished experiments). By 24 hr. there were occasional hepatic cell foci, of the size that occurs 13 hr. after massive injections. This suggests that with small doses there is a delay in the release of virus from littoral cells, a delay which would also occur when littoral cells are infected by virus liberated into the blood in the primary viraemia (Fenner 1948) of naturally infected mice.

It is thought that the extent of the initial growth in or passage through littoral cells must be a determining influence in the growth of viruses in the liver.

SUMMARY

When mice die after massive intravenous injections of ectromelia virus, nearly all the virus is present in the liver. Only a very small proportion appears in the blood, bile, or hepatic lymph glands.

Growth in the liver after massive intravenous injections of virus has been analysed by the fluorescent antibody technique. There is an initial growth cycle

in some of the littoral cells, which then infect neighbouring hepatic cells. These in turn infect more hepatic cells after each cycle of growth. When, 26–30 hr. later, there have been at least three stepwise increases in the size of the infected foci, there are almost no uninfected liver cells, and mice die.

Degenerate cell fragments appear in the peripheral circulation at the time of sickness.

Hepatic cells are not infected except by littoral cells.

It is a pleasure to acknowledge the competent histological assistance of John Benge.

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EXPLANATION OF PLATES

Mouse livers after massive intravenous injection of ectromelia virus. Fluorescent antibody stain. ×100.

FIG. 3.—4 hr. Small numbers of faintly fluorescent cells.

FIG. 4.—5 hr. Marked increase in number and brightness of fluorescent cells.

FIG. 5.—11 hr. First hepatic cells are now faintly fluorescent so that foci appear slightly larger.

FIG. 6.—13 hr. Fluorescent hepatic cells more numerous and brighter, so that foci are larger.

FIG. 7.—17 hr. Increase in brightness of foci.

FIG. 8.—20 hr. Foci, which are notably brighter and larger, are becoming confluent. Non-fluorescent areas still occur round central veins, for instance.

FIG. 9.—26 hr. A few non-fluorescent cells still present but most of the section is now fluorescent.

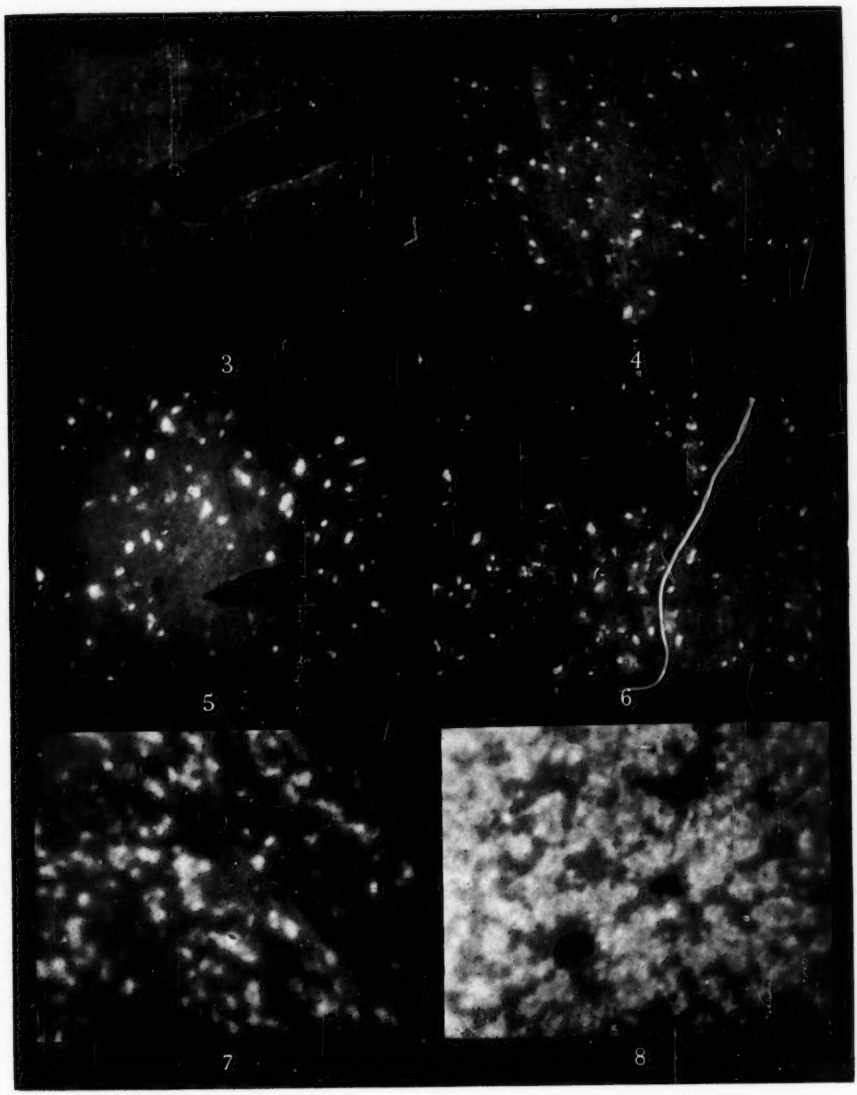
Mouse livers after massive intravenous injections of ectromelia virus. ×200–300.

FIG. 10.—11 hr. The first fluorescent hepatic cells are clearly outlined, together with the much more brightly fluorescent littoral cells. Fluorescence exclusively cytoplasmic.

FIG. 11.—16 hr. Fluorescent hepatic cell foci round a central vein. Fluorescent granules present in cells.

FIG. 12.—22 hr. Fluorescent hepatic cell foci larger and brighter.

FIG. 13.—23 hr. Fluorescent littoral and hepatic cells present.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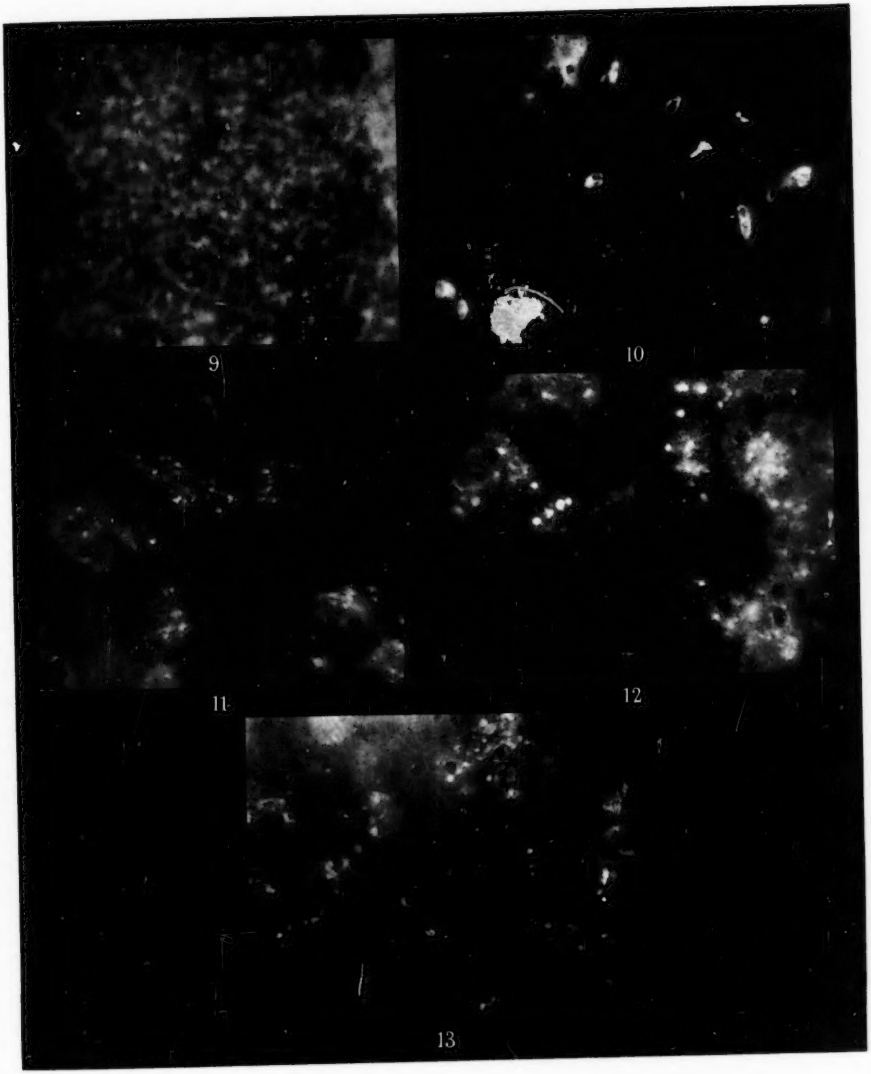

Mims.

THE INHIBITION BY DIOXAN OF ANTIGEN-ANTIBODY PRECIPITATION

I. PRECIPITATION REACTIONS WITH PROTEIN ANTIGENS

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It was shown by Grabar and Oudin (1943) that in the presence of ethanol the amount of precipitate formed in a precipitin reaction (ovalbumin and rabbit anti-ovalbumin) was increased in the region of antigen excess where normally precipitation is reduced or inhibited.

This effect would appear to depend on the reduced solubility in the presence of ethanol of the water soluble antigen—antibody complexes formed in the region of antigen excess. In this connexion, proteins may be considered as multipolar ions having large effective dipole moments. The electrostatic forces between such molecules are increased as the dielectric constant of the solvent is decreased resulting in decreased solubility. As the most likely explanation of the increased precipitation in the presence of ethanol seemed to lie in the lowered dielectric constant of the solution it was decided to study the effect of dioxan on such a system, since dioxan, like ethanol, is completely miscible with water and lowers the dielectric constant (molar decrement 7·7). It was expected that the effect of dioxan would be similar to that produced by ethanol. It was found, however, that the addition of dioxan resulted in marked inhibition of precipitation at all antigen-antibody ratios. The effect of dioxan has been studied on five precipitating systems consisting of rabbit antisera with protein antigens, and an attempt has been made to elucidate the underlying mechanism of this inhibition.

MATERIALS AND METHODS

Horse serum albumin.—This was prepared by Mr. R. G. S. Johns. It was crystallised from ammonium sulphate solution at pH 4·9 and the α_1-globulin was removed by passage through a column of powdered silica.

Crystalline bovine serum albumin and lysozyme.—These were obtained from Armour Laboratories Ltd.

Crystalline ovalbumin.—This was prepared by Mr. R. G. S. Johns according to the method of Hopkins. (1899).

γ-globulin.—Rabbit and human γ-globulins were prepared by the method of Kekwick (1940), slightly modified by Marrack, Hoch and Johns (1951) to remove small amounts of β-globulin and thus obtain a clear solution.

Rabbit antisera.—The rabbit antisera, prepared according to Proom (1943), were provided by Professor J. R. Marrack.

Dioxan.—" Analar " grade dioxan (B.D.H.) was used without further purification, the peroxide content being checked for conformity to specification. It was stored in the refrigerator. Dioxan concentrations are expressed as per cent V/V.

Protein analysis.—Protein was estimated by the method of Heidelberger and Macpherson (1943), using the Folin-Ciocalteau phenol reagent.

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Turbidity-time curves.—A convenient way of following the course of a precipitin reaction is to measure the changes of turbidity which occur when a solution of antigen is added to the antiserum (Pope and Healey, 1938). In general the turbidity rises sharply when the components are mixed, passes through a maximum and falls as the particles flocculate and settle out. Turbidities were measured in a Hilger " Uvispek " spectrophotometer at a wave length of 550 mμ. The antiserum was diluted with buffered saline (0·15 M Na Cl, 0·01 M phosphate buffer, pH 6·9) in small " Pyrex " test tubes. The amount of dioxan or alcohol required to give the desired final concentration was then added, followed by the antigen solution. After the addition of antigen the components were thoroughly mixed by inversion and the solution transferred to the measuring cell. The balancing cell contained antiserum in the same dilution as that in the measuring cell. The antisera were centrifuged before use. Generally, readings were started 1 min. after mixing. All turbidity measurements were made at room temperature.

Precipitation curves.—These were studied by adding increasing amounts of antigen to 1 ml. volumes of antiserum (diluted 1:10); the final volume was kept constant at 2 ml. by the addition of buffered saline. If tests involving dioxan were being made, a known amount was mixed with the diluted antiserum before the addition of the antigen. The tubes were allowed to stand overnight at room temperature, and at 0° for a further 48 hr. The precipitates were then centrifuged and the supernatants removed. Before analysis, the precipitates were washed twice by suspending in cold saline and centrifuging. In some experiments the antigen-antibody ratio was fixed and the amount of dioxan varied.

Dialysis experiments.—Solutions of antigen or antiserum containing dioxan (25 per cent) were allowed to stand at room temperature for the desired time and then dialysed against buffered saline. The saline was stirred mechanically and frequently changed for a period of 48 hr., after which the solutions were tested with the corresponding antiserum or antigen.

RESULTS

Normally when antigen and antiserum are mixed in the correct proportions there is a rapid increase in turbidity which is followed by flocculation and precipitation. In all the cases studied, the addition of dioxan resulted in a marked reduction in the rate of increase of turbidity and the flocculation times were greatly increased. For the horse serum albumin—rabbit antiserum system this is clearly shown in Fig. 1, the mixture containing 20 per cent dioxan remaining clear for many hours. By way of contrast, the addition of ethanol (10 per cent) caused an increase in the rate of development of turbidity and there was a slight reduction of the flocculation time.

The total amount of precipitate formed was also reduced in the presence of dioxan. The degree of inhibition varied with the antigen-antibody ratio and was greatest in the zone of antigen excess as shown in Figs. 2 and 3. It may be noted that the reaction of rabbit antiserum with lysozyme was apparently much more sensitive to the action of dioxan than the corresponding reaction in which the antigen was bovine serum albumin.

When the concentration of dioxan was increased in a system containing antigen and antibody in fixed ratio (equivalent amounts), the amount of precipitate decreased continuously and with 20 per cent dioxan, inhibition was almost complete (Fig. 4). The degree of inhibition was greatest with a dioxan concentration above 15 per cent; below this level the amount of precipitate was only slightly reduced. Under these conditions, a small increase in the amount of antigen caused a large increase in the degree of inhibition (Fig. 5).

It was found that the degree of inhibition depended somewhat on the pH of the solution. Fig. 6 shows the results obtained at pH values of 6·1 and 8·0 using a solution of rabbit γ-globulin containing antibody to bovine serum albumin. In the absence of dioxan the amount of precipitate formed was not affected by

this change of pH. When separated γ-globulin was used in place of whole antiserum, the effect of dioxan on the final amount of precipitate formed was rather less. The velocity of the reaction as indicated by the rate of increase of turbidity was, however, greatly diminished.

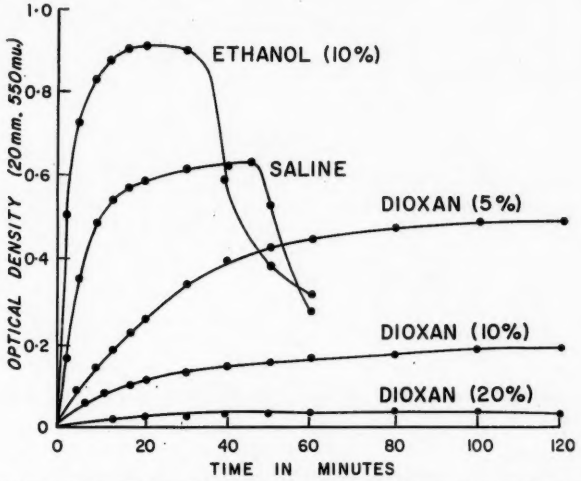

FIG. 1.—Comparison of the effects of dioxan and ethanol on the reaction of horse serum albumin and rabbit antiserum at the equivalence point.

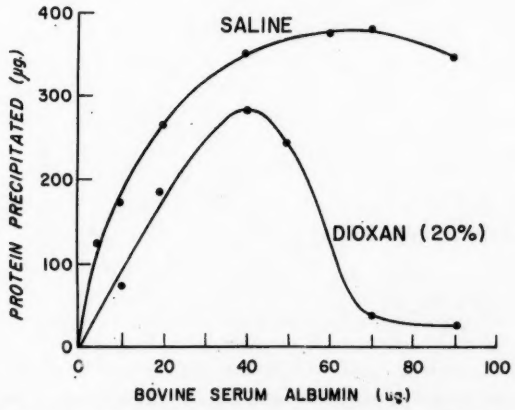

FIG. 2.—Precipitation curves for the bovine serum albumin-rabbit antiserum system with and without dioxan.

None of the antigens appeared to be denatured by treatment with 25 per cent dioxan for 4 hr. at room temperature and pH 6·9. When the dioxan was removed by dialysis and portions of the treated antigen solutions were tested with the corresponding antisera, rapid precipitation occurred. The antisera, however,

showed changed properties after similar treatment, and in all cases when these were tested with the appropriate antigens, precipitation was delayed as compared with untreated antisera. Partially purified antibody (γ-globulin) to bovine serum

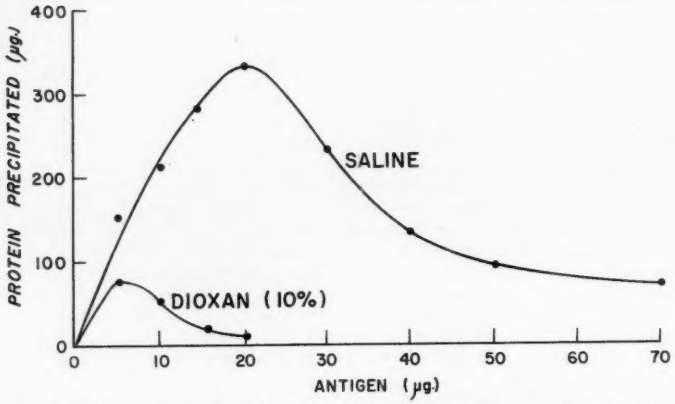

FIG. 3.—Precipitation curves for the lysozyme-rabbit antiserum system with and without dioxan.

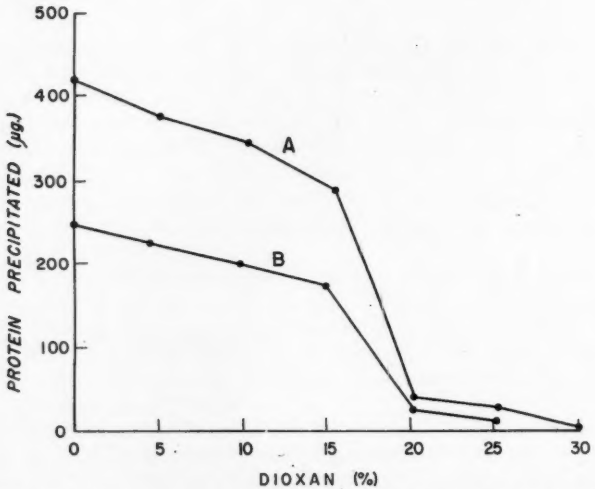

FIG. 4.—The effect of increasing dioxan concentration on the amounts of precipitate formed by rabbit antisera added to equivalent amounts of A: bovine serum albumin; B: human γ-globulin.

albumin appeared to be less affected in this respect than the whole antiserum, for it precipitated rapidly with antigen after removal of the dioxan by dialysis. The effect of dioxan appears to be largely reversible for when a mixture of antigen, antiserum and dioxan showing no sign of reaction was dialysed against saline,

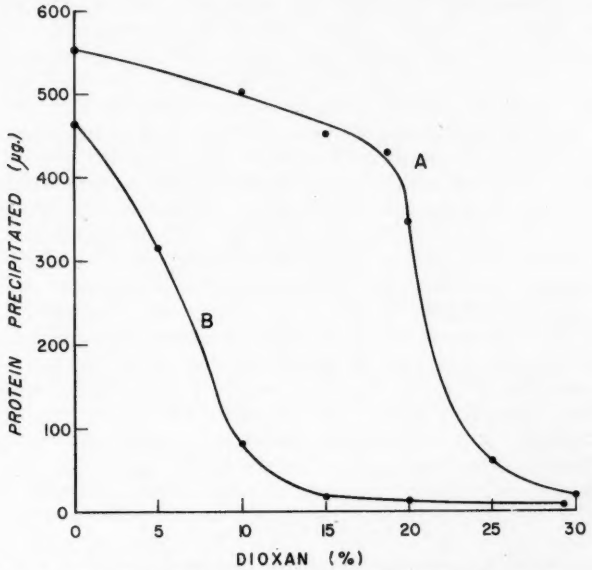

FIG. 5.—The effect of dioxan on the ovalbumin-antiovalbumin system. A: 1 equivalent of antigen; B: 1·25 equivalents.

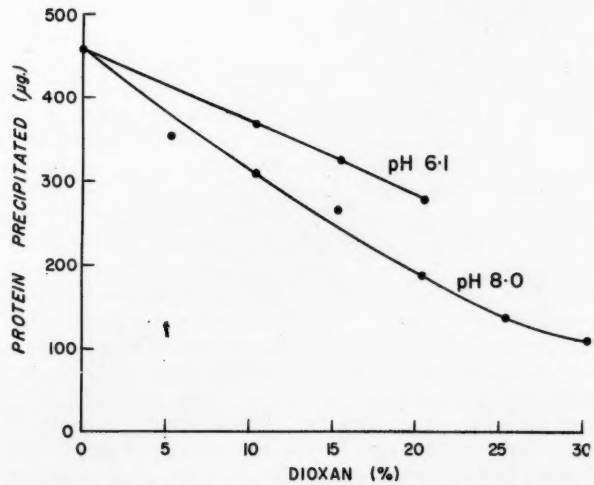

FIG. 6.—The effect of change of pH on the degree of inhibition of the reaction of equivalent amounts of rabbit antibody (γ-globulin) and bovine serum albumin.

the turbidity increased and precipitation occurred as the dioxan diffused out of the sac.

DISCUSSION

In view of the mild experimental conditions and the unreactive nature of dioxan, it is unlikely that the inhibitory effect on antigen-antibody precipitation involves the formation of covalent chemical bonds.

The presence of dioxan resulted in a decrease in the overall reaction rate indicated by the slower development of turbidity. There was also a decrease in the extent of reaction as shown by the smaller amount of precipitate.

Although dioxan lowers the dielectric constant of the solution, the above effects do not appear to depend on this, since ethanol, which also lowers the dielectric constant, does not inhibit. Ethanol increases the rate of turbidity development, and as shown by Grabar and Oudin (1943) it increases the amount of precipitate in the zone of antigen excess where dioxan inhibits most strongly.

Dioxan is capable of forming hydrogen bonds to molecules possessing hydroxyl groups, as demonstrated by its association with water molecules (Hasted, Haggis and Hutton, 1951). It has also been found that compounds such as ethylene glycol and glycerol, which are highly associated through hydrogen bonds, exert inhibitory effects on precipitin reactions (Grant, 1953). It may be noted that gamma-globulin contains relatively large amounts of serine (11·4 per cent) and threonine (8·4 per cent). Both of these amino acids possess hydroxyl groups which can participate in intermolecular hydrogen bonds, other such groups being the hydroxyl group of tyrosine, and carboxyl and imino groups. The combination of antigen with antibody is considered to depend mainly on Van der Waals interactions, ionic bonds and hydrogen bonds (Pauling, Campbell and Pressman, 1943). The energy of formation of hydrogen bonds is about 5 Kcal. per mole, this being of the same order of strength as the attraction between isolated charges in water. If dioxan combines with proteins by the formation of hydrogen bonds, it might be expected to inhibit the combination of antigen and antibody, firstly, by preventing the close approach of the specific surfaces—a necessary preliminary step—and secondly, by blocking specifically arranged hydrogen bonding centres. As the effect of dioxan was greatest in the region of antigen excess, it would appear to combine mainly with antibody. The combination of dioxan with protein is apparently reversible, for when the dioxan was removed from a mixture of antigen and antibody by dialysis, precipitation occurred. In the case of the antigens, the results of the dialysis experiments showed that exposure to 25 per cent dioxan did not permanently alter these substances. Antisera similarly treated showed a loss of precipitating power, but separated gamma globulin was affected to a much smaller extent. These results may be compared with the findings of Cohn and Pappenheimer (1949) who found that human antitoxic sera heated at 56° for a short time did not precipitate with antigen. However, when separated gamma globulin containing antitoxin was similarly treated, the ability to precipitate was not lost, but when mixed with crude serum albumin it again became sensitive to heat. These effects may be due to the formation of complexes between antibody and other serum proteins.

The effect of change of pH on the degree of inhibition probably depends on the sign and magnitude of the electrical charges carried by the antigen and antibody molecules. At pH 6·1 the antigen (I.P. 4·8) and antibody (I.P. 6·6) molecules

are oppositely charged, and will tend to attract one another; hence the degree of inhibition should be less at pH 6·1 than at pH 8·0. In the latter case the molecules carry charges of the same sign, resulting in mutual repulsion. The pH effect should increase as the percentage of dioxan rises, since electrostatic forces become greater as the dielectric constant of the medium is reduced. The results shown in Fig. 6 appear to be in accordance with this argument.

Germane to this discussion is the recent finding by Fredericq (1957) that in 20 per cent dioxan the dissociation of insulin was strongly enhanced, and in 40 per cent dioxan it was almost completely dissociated over a wide range of concentration. Also, Spar, Bale and Goodland (1957) found that urea (16–32 per cent) and sodium salicylate (10 per cent), both of which are strong hydrogen bonding agents, dissociated antigen-antibody precipitates without denaturing the antibody. This is supported by the work of Friend (1953) who found that sodium salicylate had an inhibitory action on the formation of precipitates by rabbit antisera to egg albumin and Type III pneumococcus. The finding of Kleinschmidt and Boyer (1952) that 2 M urea inhibited the precipitation of soluble proteins by their specific antisera, and also dissolved preformed precipitates, provides additional evidence for the importance of hydrogen bonding in this type of reaction. Since dioxan at concentrations above 30 per cent precipitates proteins from solution, it does not seem probable that the inhibitory effect is due to increased solubility of the antigen-antibody complexes.

SUMMARY

Dioxan has been shown to exert an inhibitory effect on five precipitin systems in which protein antigens reacted with rabbit antibodies.

The degree of inhibition increased with the antigen-antibody ratio.

The mechanism of inhibition appears to depend on the hydrogen bonding properties of dioxan rather than on the lowering of dielectric constant.

Dioxan appears to affect both the rate and extent of reaction.

None of the antigens was denatured by treatment with 25 per cent dioxan for 4 hr. at room temperature.

Whole antiserum appeared to be much more sensitive to the denaturing effect of dioxan than separated γ-globulin.

The degree of inhibiton varied with the pH of the solution.

The author wishes to thank Professor J. R. Marrack for his advice and encouragement.

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ENZYMATIC PURIFICATION OF ANTIDIPHTHERIA AND ANTITETANUS SERA OBTAINED FROM SHEEP

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In spite of careful enzymatic purification the antitoxic horse sera now used prophylactically or therapeutically still contain species specific protein. Consequently these products may sensitize the recipient to horse protein and cause hypersensitivity reactions when a further injection is given. It is therefore desirable that antitoxic sera should also be prepared from other species.

In this Institute we have succeeded in producing sheep sera which can be used therapeutically as well as prophylactically, thanks to their high antitoxin content after purification.

Until recently we purified antidiphtheria serum from sheep by salting-out only. The resulting final product however contained little antitoxin in proportion to the total protein content, while all the species specific properties were retained (van Triet, 1957). Enzymatic treatment as described by Pope (1938, 1939*a*, *b*) also reduces the amount of specific protein in sheep serum very considerably, but the further routine worked out for horse serum leads to unacceptable losses of antitoxin in this case.

It must theoretically be possible to find suitable conditions for the enzymatic purification of anti-diphtheria serum from sheep also, and therefore we have investigated the optimum conditions for this process. Using the experimental technique of Box (1954) in a statistically sound design as described by van Triet and Bloemena (1956) and van Triet (1957) we succeeded (in cooperation with the Mathematical Centre at Amsterdam) in achieving our object after a total of 306 small scale experiments. After this a very limited number of experiments sufficed to show how antitetanic serum from sheep could be purified as efficiently as possible.

Purification procedure

The conditions under which the purification of antidiphtheria sera from horses and sheep is carried out are shown in the table below. Our raw material was a serum stored for some time at 4° and preserved with 0·35 per cent tricresol.

Normal routine purifications follow strictly the scheme here described, but in small-scale experiments, the special treatment of the dialysate (the ultrafiltration and the Seitz filtration) are omitted in view of the small quantities. These operations are also not essential in a comparative examination. This different treatment accounts for the relatively smaller recovery in routine purifications, as these manipulations inevitably involve some loss of antitoxin.

*With the technical assistance of W. J. van Rhee.

Synopsis of the Purification of Antidiphtheria Serum

Instructions	Values of factors for Horse serum	Values of factors for Sheep serum
Dilute 1 vol. of crude serum with 2 vol. of tap water.		
Add dilute HCl (1 : 1) and adjust the pH of the diluted serum to	3·2	3·4
Add pepsin (Zimmerman and Co.) and adjust pH if necessary	0·133 per cent	0·200* per cent
Heat at	25°	27·5°
for	30 min.	35 min.
Then add: (a) anhydrous $Na_4P_2O_7$	0·2 per cent	0·2 per cent
(b) $(NH_4)_2SO_4$	18 per cent	14·5 per cent
(c) toluene	0·8 per cent (v/v)	0·8 per cent (v/v)
Adjust the pH to	4·3	4·5
Heat mixture (thermo-coagulation) at	55°	50°
for	60 min.	65 min.
Filter off the precipitate formed (aspecific protein).		
Add to the filtrate (a) anhydrous $Na_4P_2O_7$	0·2 per cent	0·2 per cent
(b) $(NH_4)_2SO_4$	12 per cent	6·5 per cent
Adjust the pH to	5·3	5·3
Filter off the precipitate (after standing at least 1 hr.) and redissolve it by dialysis against chlorinated tap water†		
To this dialysate add: (a) NaCl	0·85 per cent	0·85 per cent
(b) a mixture of tricresol and ether (1 + 1)	0·8 per cent (v/v)	0·8 per cent (v/v)
Adjust pH to	8·0	4·5
with	NaOH (5 per cent)	citric ac.‡ (25 per cent)
After about 20 hr. settling, syphon off the supernatant and adjust pH to	7·0	7·0
Concentrate the dilute solution by ultrafiltration.		
Sterilize the final product by Seitz filtration.		
Determine the protein content and the antitoxin content.		

* Unless otherwise stated percentages are in weight per volume (w/v).
† 2 p.p.m. active chlorine.
‡ Citric acid must be used. Hydrochloric acid does not give a filterable precipitate.

Results of purification

We purified 3 different sheep sera in 2 ways, namely according to the instructions for horse serum and by the method evolved by ourselves. These experiments were performed in quadruplicate.

As appears from Table I, the process worked out for horse serum, with the help of data supplied by Pope, must be adapted before it can be applied to sheep serum.

These results have, of course, been calculated for a dialysate that had not undergone the futher operations used in routine purifications. Consequently the recoveries obtained in these experiments with the "sheep" formula are higher than those shown in Table II.

In the routine purification of horse antidiphtheria serum the method described in the previous section gives an antitoxin recovery from 45–55 per cent, the purification factor (the PF is the ratio of the amounts of antitoxin per g of protein after and before the purification.) ranging from 2·8 to 3·8. As will be seen from the figures given in Table II, the recoveries for the corresponding sheep serum are less favourable.

TABLE I.—*Comparison of Two Combinations of Factors for Purification of Antidiphtheria Serum Obtained from Sheep*

"Horse" purification process						"Sheep" purification process					
No.*	Volume dialysate (ml.)	Titre (AU/ml.)	Protein (per cent)	Recovery (per cent)	PF	No.*	Volume dialysate (ml.)	Titre (AU/ml.)	Protein (per cent)	Recovery (per cent)	PF
Starting from 100 ml. crude serum (A) containing 400 AU/ml. and 6·6 per cent protein											
A_1	200	21	0·56	10·5	0·62	A_5	200	89	0·67	44·5	2·19
A_2	200	24	0·56	12·0	0·75	A_6	200	82	0·62	41·0	2·23
A_3	200	21	0·53	10·5	0·65	A_7	200	87	0·64	43·5	2·23
A_4	200	20	0·50	10·0	0·66	A_8	200	91	0·63	45·5	2·38
			Mean	10·8	0·68				Mean	4·36	2·26
Starting from 100 ml. crude serum (B) containing 640 AU/ml. and 6·9 per cent protein:											
B_1	200	33	0·45	10·3	0·79	B_5	200	143	0·63	44·7	2·45
B_2	200	33	0·45	10·3	0·79	B_6	200	157	0·60	49·1	2·82
B_3	200	47	0·61	14·7	0·83	B_7	200	150	0·58	46·9	2·79
B_4	200	36	0·63	11·3	0·62	B_8	200	157	0·62	49·1	2·73
			Mean	11·6	0·76				Mean	47·5	2·70
Starting from 100 ml. crude serum (C) containing 700 AU/ml. and 8·2 per cent protein:											
C_1	200	55	0·58	15·7	1·11	C_5	210	180	0·88	54·0	2·39
C_2	200	50	0·58	14·3	1·01	C_6	200	180	1·00	51·4	2·11
C_3	200	55	0·60	15·7	1·07	C_7	200	177	0·87	50·6	2·38
C_4	200	45	0·61	12·9	1·03	C_8	200	180	0·90	51·4	2·34
			Mean	14·7	1·06				Mean	51·9	2·31

* For the sake of clarity the random order in which these experiments were carried out has not been stated.

TABLE II.—*Purification of Antidiphtheria Sheep Serum (on a Larger Scale)*

	Crude serum			Purified serum			Results of purification	
Batch No.	Volume (litres)	Titre (AU/ml.)	Protein (per cent)	Volume (litres)	Titre (AU/ml.)	Protein (per cent)	Recovery (per cent)	PF
1	30·0	400	8·6	2·25	1800	13·7	33·7	2·83
2	30·0	450	8·3	1·75	2600	19·5	33·7	2·46
3	30·0	380	7·6	1·60	2300	17·0	32·3	2·70
4	31·0	450	8·1	2·10	2600	18·4	39·1	2·55
5	15·0	600	7·9	1·25	3100	19·3	43·0	2·12
6	15·0	600	7·9	0·625	3000	17·5	20·8	2·26

In preliminary experiments we found that sheep antidiphtheria serum is very sensitive to too low a pH. During purification the serum of batch no. 6 was accidentally kept at pH 2·6 for a short time. This probably accounts for the low recovery when this batch was purified.

Although sheep antidiphtheria serum cannot yet be purified as satisfactorily as horse serum, the combination of factors described here has been found suitable for practical application.

Some Properties of Purified Sheep Antidiphtheria Serum

Species specificity

The great advantage of serum purified enzymatically as compared with that purified only by salting-out lies in the reduction of the amount of species specific protein. This applies to both horse serum and sheep serum. We have ascertained (starting from material containing 6·4 per cent protein) how much the preparations mentioned in Table III can be diluted so as still to precipitate (ring reaction)

TABLE III.—*Precipitation Test with Antidiphtheria Sheep Serum*

Precipitation test with antidiphtheria sheep serum	
Preparation	Marginal dilution
Crude plasma	$\frac{1}{2^{11}}$
Plasma salted out only	$\frac{1}{2^{12}}$
Enzymatically purified plasma	$\frac{1}{2}$

with an anti-sheep-protein serum obtained from rabbits. According to the figures thus found, sheep antidiphtheria serum purified only by salting-out contains twice as much specific protein as the crude product, whereas the enzymatically purified serum has retained only one thousandth part of the original specific material.

This is in complete agreement with the results for the corresponding horse sera (van Triet, 1957).

Stability

We tested the first 2 batches of purified sheep serum to see whether the antitoxin content decreased on prolonged storage. It appeared that this material is reasonably stable, provided it is kept at 4° or 18° (see Table IV). In view of a trace of precipitation at 18°, however, a temperature of 4° is to be preferred.

Apart from this, the variations in antitoxin content are no doubt partly due to inaccuracies inherent in an *in vivo* titration.

TABLE IV.—*Stability of Purified Antidiphtheria Sheep Serum*

Description	Date	Titres in AU/ml. after storage at 4°	Titres in AU/ml. after storage at 18°
Batch 1—Purified on	22.4.57	1800	—
Commencement of test on	26.6.57	1800	1800
Checked on	9.10.57	1800	1700
,, ,,	13.1.58	1800	1600
,, ,,	4.6.58	1850	1700
Batch 2—Purified on	25.4.57	2600	—
Commencement of test on	26.6.57	2600	2600
Checked on	9.10.57	2350	2250
,, ,,	13.1.58	2400	2400
,, ,,	4.6.58	2400	2300

Flocculation test

In contrast to horse serum, sheep serum produces some curious results in the Ramon flocculation reaction. With crude diphtheria toxin we observed several flocculation zones but with purified toxoid only one. With horse serum the *in vivo* and *in vitro* titres are about the same. In the case of sheep serum, however, the animal titre is considerably higher than the Ramon titre (3·5–4 times as high).

The high value here found for the " serum ratio ", has, however, no connection with the " dilution ratio "; if these products are titrated *in vivo* at different levels, using the method of Glenny and Llewellyn-Jones (1931), identical values for the antitoxin content are found.

Some titration figures referring to the foregoing are given in Table V.

TABLE V.—*Titrations of Antidiphtheria Sheep Serum*

Titration level	Titres in AU/ml. of batch No. 4 Crude	4 Purified	5–6 Crude*	5 Purified	6 Purified
Lr/10	425	2470	580	3100	2900
Lr/100	450	2600	600	3100	3000
Lr/1000	450	2600	575	3100	2950
In vitro titration	127	756	160	752	728
In vivo†/*in vitro*	3·5	3·4	3·8	4·1	4·1

* This crude serum was purified in 2 portions.
† Titre obtained by titration on Lr/100 level.

Purification of Antitetanus Serum obtained from sheep

On the basis of knowledge and experience gained in the purification of antidiphtheria serum we had no difficulty in finding the most favourable combination of factors for the purification of antitetanus serum. We found only three slight differences :

(*a*) The thermocoagulation should take place not at 50° but at 48°.

(*b*) The first salting-out must be carried out with 15 per cent ammonium sulphate.

(*c*) In the second salting-out 6 per cent of this salt must be added (for antidiphtheria serum these figures are 14·5 per cent and 6·5 per cent respectively).

Using the combination of factors thus found we purified 3 batches of antitetanus serum from sheep. The results obtained with this serum (Table VI) were much better than those obtained with antidiphtheria serum (see Table II).

TABLE VI.—*Purification of Antitetanus Serum from Sheep (on a Larger Scale)*

Batch No.	Crude serum Volume (litres)	Titre (AU/ml.)	Protein (per cent)	Purified serum Volume (litres)	Titre (AU/ml.)	Protein (per cent)	Results of purification Recovery (per cent)	PF
1	32·0	400	7·5	2·50	2800	21·7	54·7	2·42
2	31·0	580	7·8	2·55	3500	16·6	49·6	2·83
3	34·0	850	7·4	2·30	6000	19·7	47·7	2·65

It should be remembered, however, that a difference of this kind in the purification of antidiphtheria and antitetanus serum is also found with horse serum.

Some Properties of Purified Antitetanus Serum Obtained from Sheep

Stability

The purifications described above are of such recent date, that no figures on stability in storage are available as yet. However, by analogy with the antidiphtheria serum, this preparation may be expected to have a satisfactory stability.

Flocculation test

As with antidiphtheria serum, Ramon's flocculation test can only be applied to purified toxoid and here too the *in vivo* titres and *in vitro* titres differ greatly. With these products however, the "serum ratio" is more varied than with antidiphtheria serum. Here, also the animal titrations were carried out at different levels. The figures are given in Table VII.

TABLE VII.—*Titrations of Antitetanus Sheep Serum*

	Titres in AU/ml. of batch No.					
	1	1	2	2	3	3
Titration level	Crude	Purified	Crude	Purified	Crude	Purified
L +/10 . .	400	2800	580	3400	850	6000
L +/100 . .	410	2850	600	3500	900	6200
L +/1000 . .	400	2800	580	3500	850	6000
In vitro titration .	150	810	371	1750	560	3250
*In vivo**/*in vitro* .	2·7	3·5	1·6	2·0	1·5	1·9

* Titre obtained by titration on L +/10 level.

DISCUSSION

As pointed out in the introduction it is desirable to have sera from other animals available, in view of possible sensitization, when a patient has to receive a second serum injection. With respect to the injection of aspecific protein two drawbacks attach to the prophylactic and therapeutic use of serum. In the first place this aspecific protein may stimulate the formation of antibodies, which may produce anaphylactic phenomena when serum from the same species is injected again. Thus Lansberg (1950) describes a case of severe shock and Schönbauer (1955) reports a case ending fatally. In the latter case there was an interval of as much as 26 years between the 2 serum injections.

Huygen and Tasman (1958) also describe a case of severe shock after a second administration of serum (with an interval of 20 years between the 2 injections). Notwithstanding the fact that quantitatively the specific properties have been considerably reduced by the enzymatic method of serum purification now used, they are still present to a degree which may be dangerous for certain persons. Therefore purified serum must still be looked upon as an aspecific protein for man.

An *in vitro* (precipitation) test also showed that in this purification process a new, though weaker, antigen is formed, which can produce antibodies against " digested " horse protein in rabbits. This manifests itself especially after 2 sensitizing injections (van Triet, 1957). The same phenomenon has been observed by Schöttler (1956) in animal tests.

In the second place the antitoxic serum proteins may stimulate the formation of antibodies against the injected antitoxins, so that after a second injection of serum from the same species the antitoxins are soon inactivated. Thus Littlewood, Mant and Payling Wright (1954) describe the case of a patient who was twice injected with prophylactic antitetanus serum (obtained from horses), with a 6 months' interval, and who died of tetanus shortly after the second injection.

That a second serum injection may be less effective than the first had already been demonstrated in animal tests (rabbit) by Ramon and Falchetti (1935) using sera that had not been treated enzymatically, while Regamey (1944) obtained the same results with " digested " sera. It was found that as a result of an injection of antitoxic serum the next administration of serum does not raise the antitoxin content of the blood to the same level as the first, the antitoxin being moreover excreted more quickly.

This makes it highly desirable that sheep sera, of which the antigenic (species specific) properties are greatly reduced by enzymatic treatment, should be produced, in addition to horse sera.

That sheep antidiphtheria serum now also can be concentrated sufficiently to make it suitable for therapeutic use is a great practical advantage. The diphtheria patient who has previously been injected with horse serum (*e.g.* prophylactic antitetanus serum after an accident, or therapeutic antidiphtheria serum during a previous attack of diphtheria) can now be treated with sheep serum with a better chance of success, the possibility of the complications described above (hypersensitivity, inactivation of antitoxin) being practically excluded or greatly reduced.

SUMMARY

Experiments leading to an enzymatic purification process for antidiphtheria serum and antitetanus serum obtained from sheep are described. In this way we obtained a refined product containing relatively little protein and few species specific elements. At the same time the antitoxin content is so much increased by concentration that the purified sheep antidiphtheria serum can also be used therapeutically.

The practical aspects of the use of sheep sera in addition to horse sera are discussed.

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EXPERIMENTAL VENOUS THROMBO-EMBOLISM

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ALEXANDER (1954) demonstrated that convertin (Factor VII), one of the factors accelerating the conversion of prothrombin to thrombin (Alexander, 1948) was increased in the blood of women in the last three months of pregnancy and in the puerperium, and suggested that this might be the basis of some of the thrombotic complications of that period, while Wessler (1955) produced thrombosis in isolated venous segments in dogs, following the systemic infusion of serum fractions rich in convertin.

The demonstration that a temporary increase in one of the normal components of the coagulation mechanism could, in the presence of venous stasis, result in thrombosis, led Wessler (1956) to postulate a "temporary thrombotic state", based on a transient hypercoagulability of the blood, as the underlying cause of cases of recurrent pulmonary embolism in otherwise healthy young adults, while Freidman (1958) described temporary increases in the coagulability of the blood, accompanied by a rise in the serum cholesterol, in males at times of occupational stress.

This increasing interest in transient states of hypercoagulability prompted us to study thrombus formation, following the infusion of convertin-rich serum fractions, semi-quantitatively, and it was considered that the use of an occluded venous field would mimic natural conditions more closely than an isolated venous segment, and thus allow the early changes in and the embolisation of venous thrombi to be studied in more detail than has hitherto been possible.

The portal vein of the rabbit was chosen because of its accessibility, the uniformity of its anatomy and the ease with which both the retrograde extension of thrombus into its tributaries could be traced and emboli retrieved from its main branches.

MATERIALS AND METHODS

Eluates, rich in convertin, of barium sulphate extracts of dog's serum were prepared by the method described by Wessler (1955), 500 ml. of serum producing approximately 150 ml. of eluate.

Rabbits of both sexes were used, weighing between 1·5 and 4 kg. Under ether anaesthesia, the peritoneal cavity was opened through a midline incision, the mesentery spread out and the portal vein identified.

Within 30 sec. of the injection of a volume of eluate into the marginal ear vein, a bulldog clamp was placed on the portal vein just distal to its bifurcation near the liver. This clamp remained in position for a variable period, after which, with the clamp still in position, the portal vein was decompressed by opening a small tributary in order not to dislodge any small

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thrombi. Only then were the main vessel and its tributaries opened to determine the presence and extent of the thrombus.

Coagulation time.—A modification of the Lee and White method was employed, using siliconed syringes and tubes. Blood was obtained from either a common iliac vein or the inferior vena cava and, where serial samples were required, successive venipunctures were made at progressively proximal sites. On each occasion 1·0 ml. of blood was removed, of which 0·5 ml. was transferred immediately to a glass tube of 0·8 cm. internal diameter, a procedure designed to reduce the contamination of the sample. The tube was then placed in a water bath at 37° and inspected every 30 sec. until clotting had occurred.

Fibrinolysin.—The presence of massive amounts in the systemic circulation was tested for by inspecting the coagulation tubes for complete lysis of the clot after incubation at 37° for 48 hr.

Thrombin time.—This was performed using Fibrindex (Ortho Products) in a concentration of 50 units per ml.

Prothrombin time.—A one-stage method was performed using Simplastin (Warner Chilcott) as the thromboplastic agent.

RESULTS

Anatomy of the rabbit's portal vein

The main anatomy of the rabbit's portal vein is shown diagrammatically in Fig. 1. The colic, intestinal, pancreatico-duodenal and appendicular veins

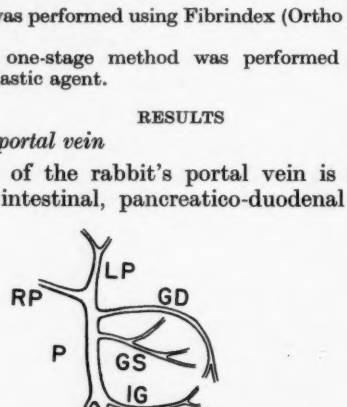


FIG. 1.—C—Colic vein. I—Intestinal vein. P.D.—Pancreatico-duodenal vein. A—Appendicular vein. S.M.—Superior mesenteric vein. I.M.—Inferior mesenteric vein. P—Portal vein. R.P.—Right branch of portal vein. L.P.—Left branch of portal vein. G.D.—Gastro-duodenal vein. G.S.—Gastro-splenic vein. I.G.—Inferior gastric vein.

unite to form the superior mesenteric vein, and extensive anastomosis exists between the lateral extremities of the vascular fields drained by these distal tributaries.

The superior and inferior mesenteric veins unite to form the portal vein, which divides, close to the right lobe of the liver, into a short narrow right branch and a wider and longer left branch, and is joined, from before backwards, by the gastro-duodenal, gastro-splenic and inferior gastric veins.

Measurement of thrombus

For the measurement of the amount of thrombus formed, the portal venous field was divided into two parts, the portal and superior mesenteric veins or " main vein " and the tributaries. Thrombus formation in the main vein, graded according to the amount of the vein occupied, ranged from four degrees, where thrombus filled the vessel, to one degree, where a quarter or less of the latter contained thrombus. Retrograde propagation into the distal tributaries was assessed similarly, four degrees consisting of complete extension into all the tributaries, whereas one degree consisted of either moderate extension into one tributary or a lesser extension into several.

The ageing and embolisation of experimental venous thrombi

Thrombus formation always began just distal to the clamp and spread backwards to fill the main vein, no extension into the proximal tributaries occurring until this was complete. The thrombi, when newly formed, resembled the post-mortem variety, being dark red and soft with a smooth glistening surface and often showing considerable elasticity. The early macroscopical changes in the thrombi and their embolisation were studied by allowing the animals to survive after clamping the portal vein.

Hardening and shrinkage of the thrombi with dulling and roughening of their surfaces could always be detected after twelve hours, but before this they were often indistinguishable from the post-mortem variety. Patchy pallor was recognisable after twenty-four hours and marked after three days, at which time the thrombi were still not adherent to the vessel wall.

The systemic infusion of 25,000 units of activated fibrinolysin (Actase, Ortho Products) dissolved in 5 ml. of saline into each of 2 rabbits, 15 min. before the injection of eluate, resulted in thrombi which were far more fragile than those produced in control animals.

Embolisation took place either totally or in stages. In the former, the whole thrombus moved on, although in one animal residual thrombus was found in the proximal tributaries, possibly having been sheared off the main mass as the thrombus embolised. After impaction in the tapering left branch of the portal vein, the proximal end of the embolus became compressed and pale, while its erstwhile extensions into the tributaries became impacted in the distal end of the main mass in which they were gradually incorporated by superadded thrombus (Fig. 2). After 4 days large emboli were still not obviously adherent to the vein wall.

When embolization occurred in stages, the extension into one or more of the distal tributaries became impacted onto the main thrombus, which still remain anchored by extensions into the other tributaries (Fig. 3).

Thrombus formation and coagulability

The blood volumes of rabbits, weighing between 1·5–4 Kg., were estimated on a basis of 6·2 per cent of the body weight (Dukes, 1947) and doses of a single eluate, varying from 0·8–10·0 ml. and designed to produce graduated concentrations in the circulating blood covering a wide range, were injected systemically. The main vein was clamped for 10 min., after which the amount of thrombus formed was assessed.

Thrombus formation was absent below a certain concentration of eluate but, as the latter was increased, became irregular in occurrence and variable in amount, until a concentration was reached above which it was constant and almost complete (Fig. 4A).

The experiment was repeated using the lower half of the previous range of eluate concentrations with the vein clamped for 20 min. (Fig. 4B). Thrombus formation first occurred and became constant and complete at lower concentrations. The whole experiment was repeated using a different eluate, coagulation times being performed on systemic blood before, injection of the eluate immediately after, and 10 min. later. A pattern of thrombus formation, essentially similar to that already seen, occurred after clamping for both 10 (Fig. 5) and 20 min. (Fig. 6) but thrombosis first appeared and subsequently became complete

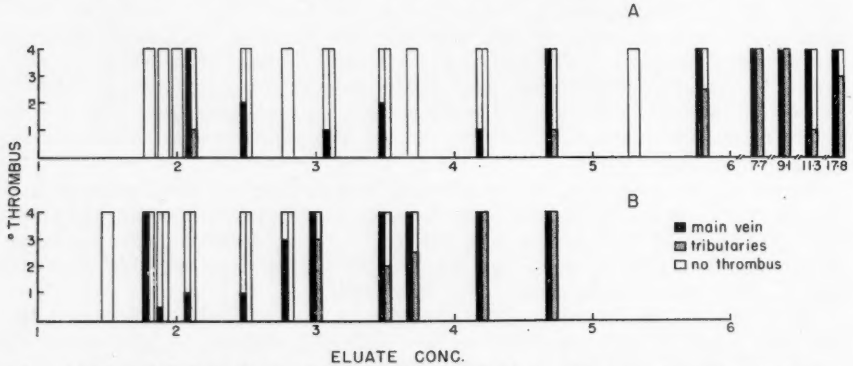


FIG. 4.—Thrombus formation in the main vein and tributaries with increasing concentrations of eluate. A—Vessel occluded for 10 min. B—Vessel occluded for 20 min.

at lower concentrations than in the previous experiment suggesting that this eluate was more potent.

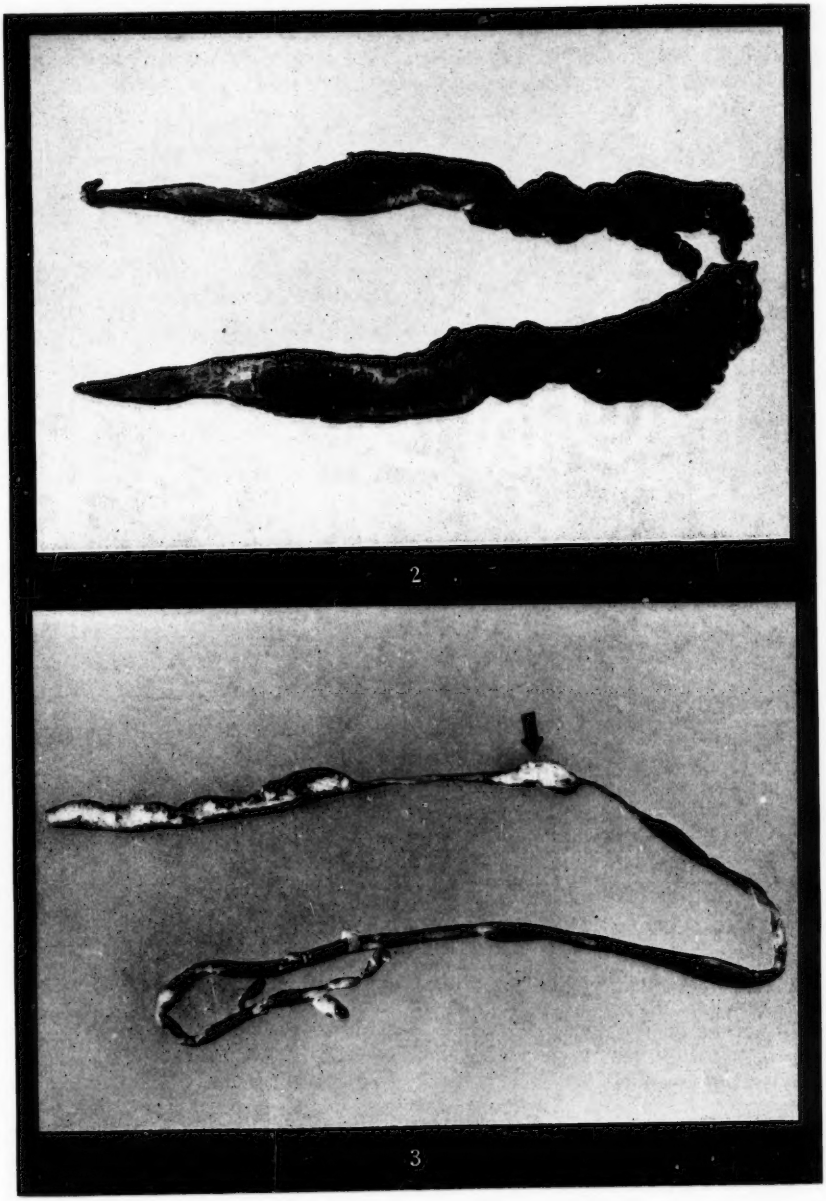
The average initial coagulation time of the 23 rabbits was 21 min., with a range of 31–6 min., 19 animals falling between 26 and 14 min. The second coagulation times were significantly shorter and, as the concentration of eluate was increased, appeared to become progressively so until a basal level of between 6 and 4 min. was reached, two animals having times of 2 min.

Saline was substituted in doses designed to produce concentrations in the blood identical to the lowest nine of circulating eluate, the range over which the progressive shortening of the second coagulation times occurred. The differences between the first and second coagulation times were far smaller than those occurring with eluate (Fig. 7), showing that this was responsible for the marked shorten-

EXPLANATION OF PLATE

FIG. 2.—Longitudinal section of 4-day-old embolus from left portal vein. Extensions are becoming incorporated into body of embolus, which shows progressive pallor from before backwards. × 2½.

FIG. 3.—Longitudinal section of 3-day-old thrombus from main vein with extension into intestinal vein. Arrow indicates possible impaction of extensions into tributaries. × 2.



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ing of the second coagulation times. The third coagulation time, performed 10 min. after injection of eluate, was shorter than the first in 17 animals, while in 5 (Fig. 5 and 6) it was prolonged. In one case (Fig. 6—No. 5) the difference was only 1 min., but in the others the prolongation ranged from 7 to 91 min.

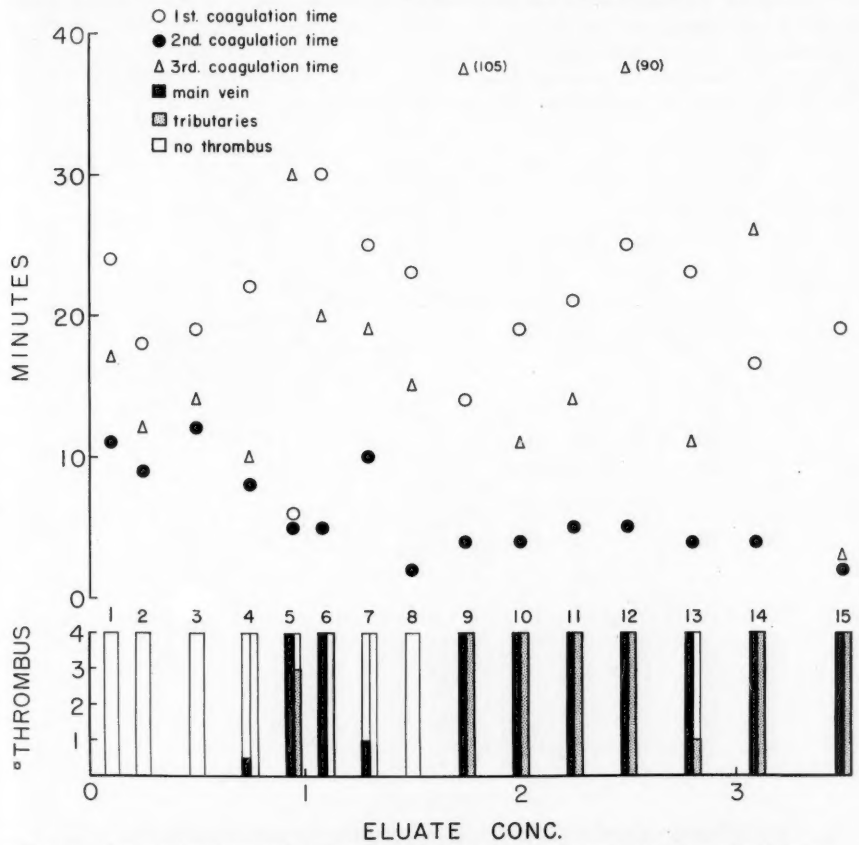

FIG. 5.—Coagulation times and thrombus formation in main vein and tributaries after 10 min. occlusion. Note prolongation of third coagulation times.

Injection of the same eluate in concentrations 50 per cent greater than any used in this series did not result in prolongation of the third coagulation time in 6 animals in which the main vein was not clamped and which consequently did not produce thrombus (Fig. 8). Histological examination of lungs, liver and kidneys of animals with thrombus, with or without prolongation of the third coagulation time, as well as of animals without thrombus formation, did not show fibrin thrombi of the type described by McKay (1958) following injection of bacterial endotoxin. Clamping the portal veins of 6 rabbits without injection

of eluate, while producing shock, did not result in prolongation of the third coagulation time. Thus this prolongation appeared to be associated, not with widespread intravascular thrombosis or the production of shock, but with the formation of massive thrombus in the occluded portal venous field only.

Incubation of the third coagulation tubes at 37° for 48 hr. did not reveal large amounts of fibrinolysin as shown by whole clot lysis. Thrombin and on stage prothrombin times, performed on samples of peripheral blood obtained at the times of the first and third coagulation times, did not show any significant alteration as the result of massive portal vein thrombosis.

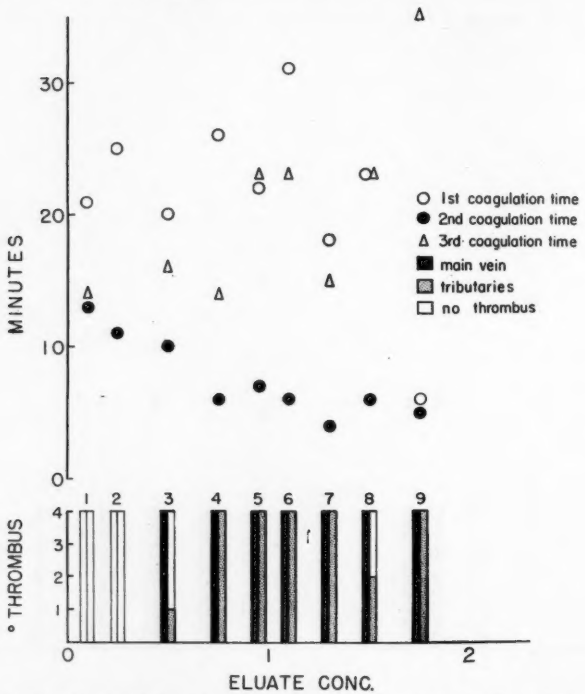

FIG. 6.—Coagulation times and thrombus formation in main vein and tributaries after 20 min. occlusion. Note prolongation of third coagulation times.

The haemodynamics of sudden portal vein obstruction

Observations were made on the haemodynamics of sudden portal vein occlusion. The portal veins of heparinized rabbits were exposed as described earlier. Two 18-gauge hypodermic needles, mounted on cerebro-spinal fluid manometers filled with normal saline, were inserted into the portal vein and a medium-sized tributary of either the colic or intestinal veins, care being taken that both needles lay in the same horizontal plane. The other distal tributaries were too small to accommodate a needle of this size. The pressure in each vessel was recorded every 30 sec. before and after clamping the main vein for as

long as the animal survived. No blood could be detected escaping through the clamp and animals in which leakage occurred at the sites of insertion of the needles were discarded.

After clamping the main vein in 8 animals, a rapid rise in pressure in both vessels was followed by a variable but progressive decompression of the portal

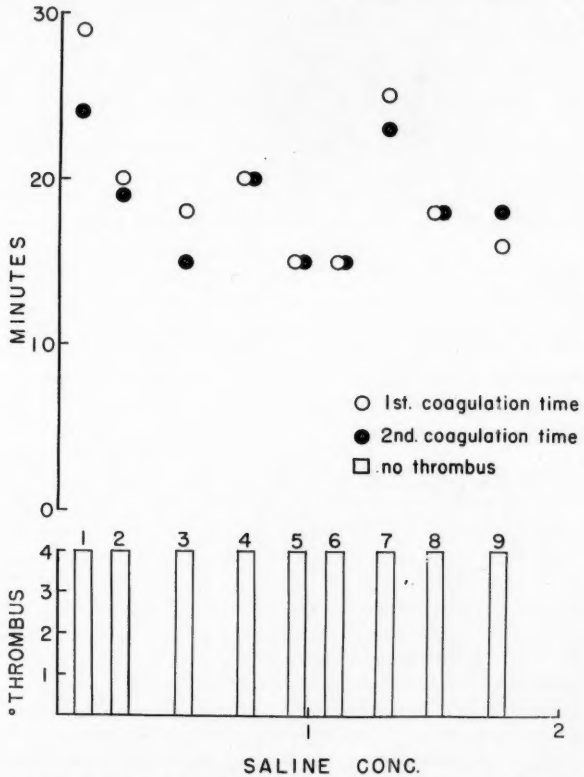

FIG. 7.—First and second coagulation times after substitution of saline for eluate.

venous field. On the basis that a fall in pressure along a vessel indicates a flow in that direction, it was seen that movement of blood persisted for a variable time after sudden occlusion of the main vein. Centripedal flow continued for periods of up to 7 min. and was usually followed by apparent stasis, but in two cases a retrograde flow supervened and continued until the animal became moribund, while an increase of pressure in a vessel, usually a tributary, during the period of decompression suggested that spasm could occur. The persistent retrograde flow in some animals suggested a collateral circulation with the systemic venous system, which might also be present to a smaller but not demonstrable extent in those cases in which stasis apparently occurred.

DISCUSSION

The growing evidence that transient hypercoagulability of the blood exists clinically is important from the point of view of not only coronary thrombosis, but also phlebothrombosis and embolism. The experimental method of inducing thrombosis, developed by Wessler (1954), was applied to the portal vein and

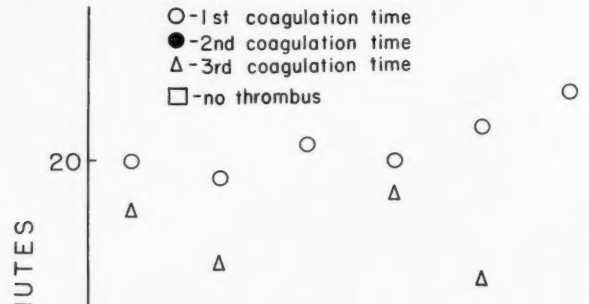

FIG. 8.—Coagulation times after injection of eluate but without clamping.

yielded direct information concerning hypercoagulability and thrombus formation in an occluded venous field, as produced by convertin-rich serum fractions, as well as the early changes in and embolisation of soft red stasis thrombi. Since the original enunciation of the 3 main factors concerned in thrombosis much of our knowledge of phlebothromboembolism has been based on indirect evidence obtained through clinical and morbid anatomical investigation.

The portal and systemic venous systems probably behave similarly after occlusion of a main vessel, despite the former's absence of valves. Edwards

(1934, 1937 and 1947) showed that in systemic thrombophlebitis dilatation of the numerous collaterals rendered their valves incompetent, permitting retrograde flow, while the decompression of the portal field, following the initial rise of pressure, resembled that reported by Fell (1955) in the calf veins after femoral thrombophlebitis.

Ante-mortem thrombi, up to 12 hr. old, were often indistinguishable from the post-mortem variety, while thrombi formed in the presence of a high fibrinolysin level were very much more fragile than normal. The original shape of a soft red thrombus may be completely altered by its impaction in a tapering vessel and by the incorporation of its distal extensions (Fig. 2), an important point when trying to determine the possible site of origin and extent of a thrombus in cases of apparently total embolisation, while both thrombi and emboli were still not obviously adherent to the vessel wall after 3 days. The similarity between the early appearances of experimental venous stasis thrombi and emboli and of those often seen in fatal phlebothromboembolism in humans suggests that many of the latter thrombi are of recent origin and embolise early.

Increasing concentrations of circulating eluate increased coagulability to a level where almost complete thrombosis of the venous field occurred in every animal, different eluates achieving this at different concentrations (Fig. 4A and 5). Doubling the period of vascular occlusion resulted in a decrease in the amount of eluate, and therefore the coagulability, necessary for this amount of thrombus formation (Fig. 4A, 4B, 5 and 6), suggesting an inverse relationship between these 2 factors, a relationship which, because of the variable behaviour of the portal venous field after occlusion, can only be broad.

Convertin-rich serum eluates produced marked, though transient, shortening of the coagulation time, the latter often returning almost to normal in 10 min. and down to a basal level, the second coagulation times appeared to bear a broad inverse relationship to the concentration of circulating eluate. In one case (Fig. 5) an abnormally short initial coagulation time was associated with massive thrombosis using an unusually low concentration of eluate, a possible explanation of the unexpectedly large amounts of thrombus produced by some animals.

After 20 min. occlusion, most of the movement of blood had usually stopped (Fig. 4B) and, apart from the first animal, which produced massive thrombus, possibly due to an abnormally short initial coagulation time, there seemed to be a broad relationship between the concentration of circulating eluate and the amount of thrombus formed. The more potent eluate illustrated in Fig. 6 possibly produced thrombus too massively to show this relationship while the latter tended to be masked through the inhibition of retrograde extension of thrombus by movement of blood during the first 10 min. occlusion (Fig. 4A).

Using a comparatively potent eluate (Fig. 5), stages leading to complete thrombosis of the venous field were few, while with a less potent one (Fig. 4A), coagulability was built up more slowly, thrombosis of the venous field being incomplete at high concentrations. It is possible that the less its retrograde propagation, the more likely a stasis thrombus is to embolise totally following shrinkage, and that the larger number of incompletely thrombosed vessels produced by the latter eluate constitute a greater danger as potential emboli. Thus minor degrees of hypercoagulability resulting in thrombosis may be more dangerous than massive ones for this reason, particularly when accompanied by a high level of fibrinolysin.

The loss of coagulability of the systemic blood, shown by the prolongation of the third coagulation time, was not apparently associated with wide-spread intravascular thrombosis but with massive thrombus formation in the portal venous field, the proximal end of which had previously been clamped off from the remainder of the circulation. It was apparently not due to massive fibrinolysis, fibrinogen deficiency or increased anti-thrombin production, degrees of these phenomena, likely to produce this profound reduction in coagulability, not being demonstrated.

This reduction in coagulability may be due either to the production of some inhibitor substance or to a deficiency in the coagulation mechanism resulting from massive thrombosis and the cause may arise within the systemic circulation, in which event the former is the more likely, or within the portal venous field itself, when either mechanism could be responsible. In this case blood would have to escape from the portal venous field to the systemic circulation and the collateral circulation would be the only route by which this could occur. The occurrence of a prolongation of the third coagulation time in 4 of 7 animals with total or almost total thrombosis of the portal venous field after 10 min. occlusion and the probable variability of the collateral circulation suggested by the haemodynamic studies tend to confirm this.

The changes in the coagulability of the blood, following infusion of convertin-rich serum eluates and massive thrombus formation in the portal vein, are violent and rapid. Similar though milder changes, taking place more slowly, probably occur in humans. Depending on the size of the collateral circulation, massive retrograde venous thrombosis in humans may be followed by a reduction, possibly transient, in the coagulability of the blood in the remainder of the circulation, due to factors other than those investigated here, this reduction limiting further thrombosis.

SUMMARY

Using the portal vein of the rabbit, thrombus formation in an occluded venous field and the hypercoagulability of the blood produced by convertin-rich serum eluates were investigated semi-quantiatively, and the early changes in and embolization of stasis thrombi studied.

The latter were only readily distinguishable from the post-mortem variety after 12 hr. and their shape became radically altered as the result of embolization, while evidence suggests that in many cases of fatal phlebothromboembolism in humans, the thrombi are of very recent origin and embolise early.

Convertin-rich serum eluates reduced the coagulation time and, down to a basal level, the latter was apparently broadly inversely proportional to the circulating concentration of the former.

The inverse relationship, in thrombus formation, between the period of vascular occlusion and coagulability was demonstrated, as well as the inhibition of retrograde propagation of thrombus by movement of blood in an occluded venous field.

The amount of thrombus formed appeared broadly proportional to the concentration of circulating eluate, initially abnormally coagulable blood producing massive thrombosis with unusually low concentrations.

A minor degree of hypercoagulability, resulting in thrombus formation, may be a greater danger as a producer of potential emboli than a massive one, parti-

cularly when associated with a high level of fibrinolysin, the latter resulting in the production of unusually fragile thrombi.

Reduction in coagulability of the blood in the remainder of the circulation, following massive thrombosis in an occluded venous field and possibly related to the presence and size of the collateral circulation, suggested a mechanism whereby thrombosis may be temporarily self-limiting.

This experimental method has not only enabled thrombus formation and coagulability to be studied directly and quantitatively but also provides a means for the further and closer investigation of allied problems.

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FURTHER OBSERVATIONS ON THE NATURE OF SOME ANTIBODY-LIKE ACTIVITIES IN NORMAL HUMAN SERA

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SPECIFIC and non-specific antibody-like activities against starch and a number of bacteria have been described in normal guinea-pig, rabbit and human sera by means of the immune-adherence phenomenon (Turk, 1959). The non-specific activity was found to be in the euglobulin fraction and had many properties in common with properdin (Pillemer *et al.*, 1954). The specific activity, which was found mainly in the pseudoglobulin, was stable to heating at 55° for 20 min. in guinea-pig and rabbit sera. In human sera however the activities were found to be mainly heat labile.

In the present paper this investigation is continued with a view to determining whether any further information can be obtained as to the nature of these activities by fractionation of normal human sera with ethanol in the cold (Cohn *et al.*, 1950) and by ultra-centrifugation.

As it has been shown that treatment of human sera with sulphydryl compounds will abolish the activity of certain bacterial agglutinins, the isohaemagglutinins and properdin (Grubb and Swahn, 1958, Isliker 1958), it was thought that treatment of normal human sera with cysteine might throw some light on the nature of the antibody-like activities demonstrated by use of the immune-adherence phenomenon.

MATERIALS AND METHODS

Antigens, sera used as a source of complement, diluent, and erythrocytes for immune-adherence titrations were prepared as described by Turk (1959).

Sera from 8 human subjects (5 male, 3 female) within the age range 17–45 with no previous history of inoculation with the antigens used in the immune-adherence titrations (except one who had received 2 injections of T.A.B. vaccine 5 years previously) were used within 24 hr. of withdrawal and separation, and stored at −70° during this time.

Purified properdin was prepared from human serum by the method of Pillemer *et al.* (1956).

Immune-adherence titrations were carried out according to Turk (1959). Titrations of properdin were performed by the modified method of Howard *et al.* (1958), using RP kindly given by Dr. Robert B. Pennel of the Blood Characterisation and Preservation Laboratory of the Protein Foundation, Inc., Jamaica Plain, Massachusetts, and zymosan obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. Erythrocyte agglutination titrations were performed according to Dunsford and Bowley (1955).

Fractionation of serum according to Cohn's method 10 (1950) was used with the modifications of Russ *et al.* (1951). Sera were fractionated in 20 ml. and 5 ml. amounts. The fractions were named in accordance with the suggestions of Russ *et al.* (1951). The composition of fractions of a typical serum are shown in Table I. A′ and B′ are the fractions removed by washing the paste after steps A and B with the reagents described by Lever *et al.* (1951).

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TABLE I

Fraction: Cohn *et al.* (1950)	Fraction: Russ *et al* (1951)	Protein content (mg./ml.)	Electrophoretic components: Major	Electrophoretic components: Minor
IV, V, VI	A	48·6	Albumin	α_1, α_2 β-globulin
	A′	4·7		
II	B	9·2	γ-globulin	β-globulin
	B′	3·8		
III_0	C	7·5	α_2-globulin	β-lipoprotein
III_{123} + I	D	9·0	β-globulin	γ-globulin

Fraction C + D, (fractions C and D together) or D pastes were dissolved in phosphate buffer pH 7·4, I. 0·2 as soon as they were recovered, and were titrated immediately. The other fractions were dialysed overnight at 4° against phosphate buffer pH 7·2, *I.* 0·2 before estimating the protein content or titrating for immune-adherence activity.

Protein estimations were by micro-Kjeldahl. Electrophoresis on paper was performed in a barbiturate buffer pH 8·6, *I.* 0·05 with a current of 0·2 ma./cm. for 16 hr.

Ultracentrifugation was performed in a Spinco model L ultracentrifuge using the 40 rotor head. The rotor temperature was maintained at 6± 2°. Fraction C + D, dissolved in a volume of phosphate buffer equal to the initial volume of the serum from which it was derived, was centrifuged at 15,000 r.p.m. for 15 min. to remove any denatured protein. The supernatant was then centrifuged at 40,000 r.p.m. (approx. 100,000 *g.*) for 8 hr. A small transparent gelatinous sediment was obtained, containing ~25 per cent of the original protein. The tubes could be inverted and allowed to drain without any loss of sediment. The sediment was brought back to volume by the addition of the phosphate buffer.

Ultracentrifugal analysis of fraction C + D by Dr. P. A. Charlwood of the National Institute for Medical Research showed three components of S_{20} ~ 3·5, 6·5, and 16. After ultracentrifugation at 40,000 r.p.m. for 8 hr. at 6° there was enrichment of the S16 component in the pellet from ~ 10 per cent to ~ 30 per cent of the protein present; the pellet also contained ~ 14 per cent of the total S6·5 protein present in the original fraction.

Treatment of sera with cysteine

Sera were diluted 1 : 10 in phosphate buffer pH 7·4, *I.* 0·2, containing 0·1 M cysteine, and left overnight at room temperature. They were then dialysed for 18 hr. at 4° against the phosphate buffer containing 0·02 M iodoacetate. A control sample of serum diluted 1 : 10 in the phosphate buffer without cysteine was also left at room temperature overnight and then dialysed for 18 hr. against iodoacetate in the phosphate buffer, in the cold. The two serum samples were titrated in parallel.

RESULTS

Comparison of the immune-adherence titres of a normal serum and the fraction D prepared from it shows that all the immune-adherence antibody-like activity against *Salmonella typhi*, *Shigella flexneri*, *Bacterium coli*, *Vibrio el tor*, *Staphylococcus aureus* and starch, as the anti A iso-haemagglutinins, are present in this fraction (Table II). No immune-adherence

TABLE II.—*Comparison of Titres* (1 :) *of immune-adherence Activity and Anti-A Haemagglutinins in Fraction D and the Serum from which it was Derived*

Antigen	Serum	Fraction D
Salm. typhi	32	32
Sh. flexneri	64	64
Bact. coli	64	64
V. el tor	256	256
Staph. aureus	64	64
Starch	256	256
Group A erythrocytes	128	128

activity was found in fractions A, A′, B, or B′. In 1 of the 8 sera tested there was immune-adherence activity present in fraction C as well as D; in this case activity was found to titre 1/6 only against *Bact. coli*, *V. el tor*, and *Staph. aureus*, and 1/24 against starch; there was no detectable fall in the titre of activity in fraction D as compared with that in the original serum.

Ultracentrifugation

Fraction C + D of six of the sera fractionated were ultracentrifuged at 40,000 r.p.m. for 8 hr. at 6°. Immune-adherence activity, properdin and the isohaemagglutinins were titrated in the supernatant and pellet immediately after removal from the centrifuge. Fraction C + D stored at 4° during the period of the centrifugation was titrated in parallel. Table III shows a typical result. The pellet contained 88 per cent of the isohaemagglutinins.

TABLE III.—*Comparison of Titres* (1 :) *of Immune-adherence Activity and Anti-A Haemagglutinins in Fraction C + D and in the Supernatant and Pellet after Ultracentrifugation at* 40,000 *r.p.m. for* 8 *hours at* 6° *C.*

	C + D	Supernatant	Pellet
Protein content (mg./ml.)	14·4	11·2	3·2
Antigen—			
Salm. typhi	32	16	4
Sh. flexneri	128	32	16
Bact. coli	64	32	16
V. el tor	32	16	4
Staph. aureus	64	32	8
Starch	256	64	32
Group A erythrocytes	256	32	256

and all the properdin activity as titrated by the method of Howard *et al.* (1958). The supernatant contained 25–50 per cent of the immune-adherence activity and there was around 12 per cent of activity in the pellet, which contained ~ 14 per cent of the protein of S_{20} 6·5 present in the original fraction. Properdin, which is known to be a heavy globulin, was found in the pellet by the conventional technique of Howard *et al.* (1958), but there was little comparable immune-adherence activity. However there was a fall in titre in the supernatant of the same order as that found after absorption with zymosan. The addition of the supernatant to the pellet was found to restore full immune-adherence activity as found in the original fraction C + D titrated in parallel. It was also found that a preparation of purified properdin, prepared by the method of Pillemer *et al.* (1956), of the same titre as the serum from which it had been derived, when titrated by the method of Howard *et al.* (1958), had no immune-adherence activity in a system using absorbed guinea-pig serum as a source of complement. This would therefore suggest that ultracentrifugation removes a co-factor from the properdin, similar to that demonstrated by Pensky *et al.* (1958), which remains in the supernatant when the properdin is sedimented. The same co-factor also appears to be removed in the preparation of purified properdin; it seems however to be present in the reagent RP but not in the absorbed guinea-pig serum used as source of complement in immune-adherence titrations.

The effect of cysteine on the immune-adherence activity of normal human serum.

Table IV shows the effect of the treatment of normal serum with cysteine followed by dialysis against iodoacetate. The immune-adherence activity against *Salm. typhi*, *Sh. flexneri*, *Bact. coli* and starch is completely abolished as also are the anti A isohaemagglutinins. There is however only a 2-fold fall in titre of activity against *Staph. aureus* and no fall in titre against *V. el tor*. This would be consistent with the survival of the specific antibody-like activities against these 2 bacteria, despite the destruction of properdin (Isliker, 1958). It has already been shown (Turk, 1959) that part of the activity against the antigens used in these experiments is specific in each case and not due to properdin. It would therefore appear in this serum that the specific antibody-like activities against *Salm. typhi*, *Sh. flexneri*, *Bact. coli* and starch were susceptible to treatment with cysteine. Similar

TABLE IV.—*Titres* (1 :) *of Immune-adherence Activity and Anti-A Haemagglutinins in serum with and without Treatment with Cysteine Followed by Dialysis Against Iodoacetate*

Antigen	Serum titres	
	Without treatment with cysteine	After treatment with cysteine
Salm. typhi	40	<10
Sh. flexneri	80	<10
Bact. coli	80	<10
V. el tor	160	160
Staph. aureus	80	40
Starch	40	<10
Group A erythrocytes	80	<10

results were found with seven other sera, except that in some cases the activity against starch also survived cysteine treatment. No activity was returned after dialysis against iodoacetate.

DISCUSSION

The total immune-adherence antibody-like activity of normal human serum, both specific and non-specific, is present in that fraction of serum left by the removal of albumin and most of the α and β globulins. As in all but one serum there was no activity in fraction C it does not seem to be a β lipoprotein. Lever *et al.* (1951) showed that, if an excess of fraction II γ globulin was added to serum, 10 per cent would appear in fraction III + I, so that if no activity is detected in fraction II (B and B′) it is very unlikely to be in the γ globulin though there are always traces of these proteins in III + I. It would therefore seem probable that the activities described are properties of the β globulins in this fraction.

A number of immune antibodies are found mainly in Cohn's fraction II, these include antibodies against viruses, diphtheria antitoxins *Salm. typhi* "H" agglutinins (Enders, 1944) and antibodies against dextran (Humphrey and Turk, unpublished observations). Enders (1944) however found that *Salm. typhi* "O" agglutinins were almost all in fraction III. The reason for the appearance of antibodies in different fractions is unknown and the results cannot be taken to indicate any basic difference between immune and natural antibodies.

The results of ultracentrifugation suggest that there is a major component of the immune-adherence activity of serum present in the S 6·5 component of the globulin. This is in contrast to properdin and the anti-A isohaemagglutinins which are in the heavy globulin fraction. As these activities are independent of properdin, it would seem probable that they are the specific activities not absorbed by zymosan, described previously (Turk, 1959).

Treatment of serum with cysteine followed by dialysis against iodoacetate completely destroys activity against some antigens. Activity against other antigens is only slightly reduced by cysteine, which would be consistent with the destruction of properdin (Isliker, 1958), leaving the specific antibody-like activity unaffected or only slightly reduced. Activity is not restored after inactivating the cysteine by dialysis against iodoacetate.

Similar findings of the effect of sulphydryl compounds in abolishing the activity of the red cell isoagglutinins, some immune antibodies, the Paul Bunnel antibody and the rheumatoid arthritis factor have been described by Grubb and Swahn

(1958), who found that these activities were not restored by dialysis against iodoacetate. It would therefore appear that sulphydryl compounds could act by reducing the disulphide linkages which, as has been suggested by Karusch (1957), may play an essential part in the maintenance of the specific configuration of the combining region of some antibody molecules.

SUMMARY

Fractionation of normal human serum with cold ethanol (Cohn's method 10) indicates that all the immune-adherence antibody-like activity resides within Cohn's fraction $III_{123} + I$ and is probably in a fraction of the β globulin. The specific activities appear to lie within the globulin fraction of S_{20} 6·5. Treatment of serum with cysteine abolishes all the activity against some antigens, whereas the activity against others is only reduced, consistent with a destruction of properdin but not of specific antibody.

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CYTOPLASMIC LOCALIZATION OF " COMPLEMENT-FIXING " AUTO-ANTIGEN IN HUMAN THYROID EPITHELIUM

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THE sera of most patients with chronic thyroiditis contain auto-antibodies directed against the thyroglobulin in the colloid of thyroid acini (Roitt, Doniach, Campbell and Hudson, 1956 ; Witebsky, Rose, Terplan, Paine and Egan, 1957 ; White, 1957 ; Doniach and Roitt, 1957) and against another constituent present in the " microsomal " fraction obtained by differential centrifugation of thyroid homogenates (Roitt and Doniach, 1958 ; Belyavin and Trotter, 1959). With the majority of Hashimoto sera, the 2 auto-antigens can be distinguished by the fact that the " microsomal " antigen-antibody reaction leads to complement fixation while only occasional sera can deviate complement with thyroglobulin (Anderson, Goudie and Gray, 1959 ; Belyavin and Trotter, 1959).

The presence of complement-fixing activity in the microsome fraction, which is particularly high in preparations from thyrotoxic glands, suggests an intracellular localization in the thyroid epithelium, but the evidence for this is not conclusive since the fate of stromal elements, basement membrane, etc. on fractionation of homogenates cannot be predicted and further the possibility of adsorption of soluble constituents of the colloid onto the subcellular granules cannot be excluded. It seemed that the problem could be resolved by the application of Coons' fluorescent antibody technique using high titre complement-fixing Hashimoto sera. The present experiments show that these sera are able to stain the cytoplasm of thyroid acinar cells specifically.

MATERIALS AND METHODS

Sections of fresh frozen thyroid glands were treated with sera from patients with thyroid disease. Tissue fixation of antibody globulin was located by the method of Coons and Kaplan (1950) using fluorescein-labelled rabbit anti-human-globulin serum.

Sera.—Sera from 6 cases of thyroid disease were investigated. The table shows the diagnosis in each case and the results of testing each serum for antibody to thyroglobulin by precipitation (Doniach and Roitt, 1957) and tanned red-cell agglutination (Roitt and Doniach, 1958), and also for the antibody to the " microsome " fraction of thyroid homogenates by complement fixation (Roitt and Doniach, 1958).

Thyroid tissue.—Normal human thyroid tissue was obtained from 2 cases of neonatal death, and toxic thyroid tissue from 2 patients at subtotal thyroidectomy. Cynomologus monkey and rabbit thyroids were removed from freshly killed animals. These unfixed tissue samples were rapidly frozen, and 6 μ sections cut in a cryostat at —20°.

Other tissues.—Normal human kidney and liver were obtained from one of the cases of neonatal death.

Treatment of sections.—Sections were thawed on to slides and dried rapidly at room temperature. In order to prevent a slight non-specific uptake of human serum protein, which sometimes occurred during treatment with normal serum, the sections were first covered with 2–3 drops of normal rabbit serum, left at room temperature in a moist chamber for 30 min. and subsequently washed in buffered saline (pH 7·0) for 5 min. The sections were then treated in the moist chamber with 2–3 drops of the patient's serum (30 min.) and washed in buffered saline (30 min.) before staining with fluorescein-conjugated anti-human globulin (30 min.). After a final washing (30 min.) they were mounted in buffered glycerol and examined microscopically. Sections similarly treated with normal human serum were included as controls. Each serum was also applied to sections previously fixed in absolute alcohol overnight, washed in buffered saline and pre-treated with normal rabbit serum.

Anti-human globulin fluorescent conjugates.—These were prepared as previously described (Holborow, Weir and Johnson, 1957).

RESULTS

In fresh unfixed thyroid sections the follicles were usually empty due to leaching out of colloid in the washing procedure so that staining of thyroglobulin was seldom found following treatment with anti-thyroglobulin-positive sera. Subsequent fixation and eosin staining of these sections confirmed the almost complete absence of colloid from the vesicles. This feature made it easier to observe specific fluorescent staining of the follicular epithelium in unfixed sections following treatment with patients' sera and subsequently with antiglobulin conjugate. When sections were fixed in alcohol before staining, however, the colloid was retained in the follicles. The use of alcohol as a fixative, therefore, made it possible to study the uptake of antibody globulin by the antigens of the colloid, while with unfixed sections the reactions of the follicular epithelium could be observed. These two phenomena will be described separately.

The antigen(s) of the thyroid epithelium

All 6 sera (see table) gave bright fluorescent staining of the thyroid follicular epithelium in unfixed sections of both toxic adult and normal infants' thyroid (Figs. 1, 2 and 3). In both types of gland the staining was clearly confined to the cytoplasm of the epithelial cells. In the normal infant this appeared as a ring of specifically fluorescent material surrounding the nucleus; in the toxic thyroid, where the cells are larger and columnar, the staining was correspondingly more extensive, but still confined to the epithelial cell cytoplasm. No other tissue in the thyroid showed positive staining, and controls using normal human serum were consistently negative (Fig. 4). Sections of liver and kidney obtained from one of the normal infants gave no staining with the positive sera which suggested that organ-specific antibodies rather than iso-antibodies were implicated in these phenomena. Cellular staining was unaffected by absorption with thyroglobulin (Fig. 2), but was completely abolished by absorption with the microsome fraction prepared from a thyrotoxic gland. One serum (E.J.) in which the complement fixation titre was reduced from 1/512 to 1/32 by incomplete absorption with microsomes gave epithelial staining of reduced intensity. Absorption with a microsome fraction from human liver failed to affect the ability of a serum to stain the acinar cells.

No cytoplasmic staining was obtained after pretreatment of the sections with alcohol. Parallel serological studies showed that alcohol treatment of microsome preparations led to loss of their complement-fixing activity.

TABLE.—*Staining of Human Thyroid by the Fluorescent Antibody Technique Using Chronic Thyroiditis Sera*

Patient	Age	Sex	Diagnosis	Serum absorbed with	Precipitin* test	TRC† titre	CFT‡ titre	Fluorescent staining: Normal and thyrotoxic thyroids: Colloid	Fluorescent staining: Normal and thyrotoxic thyroids: Cytoplasm	Fluorescent staining: Human liver kidney
W. E—	39	F.	Hashimoto's thyroiditis	*Nil*	+++	1,000,000	512	++++	+++	Not tested
				Thyroglobulin	−	—	512	−	+++	
C. H—	50	M.	Thyrotoxicosis merging into Hashimoto's disease	*Nil*	++	100,000	256	+++	+++	−
Ph. D—	18	F.	Atypical recurrent thyroiditis	*Nil*	−	5	512	+	+++	−
				Thyroglobulin	−	—	512	−	+++	..
J. K—	70	,,	Hashimoto's thyroiditis	*Nil*	+	10,000	256	+++	+++	−
				Thyroglobulin	−	−	256	−	++	..
E. J—	50	,,	Ditto	*Nil*	−	−	512	−	++++	−
				Thyroglobulin	−	−	512	−	++++	..
				Thyroglobulin + thyroid microsomes	−	−	32	−	++	..
L. M—	44	,,	Hashimoto's thyroiditis (? previously thyrotoxic)	*Nil*	++	100,000	512	++++	++++	−
				Thyroglobulin	−	−	512	−	++++	..
				Thyroglobulin + liver microsomes	−	−	512	−	++++	..
				Thyroglobulin + thyroid microsomes	−	−	−	−	−	..

* = thyroglobulin precipitin in agar.
† TRC = agglutination of thyroglobulin-coated tanned red cells using separate pipettes for each 10-fold dilution in the test.
‡ CFT = complement fixation against " thyrotoxic microsomal antigen ".

The antigen of the colloid

As mentioned above, it was necessary to fix sections in absolute alcohol in order to retain the colloid within the follicles. When these sections of normal and thyrotoxic thyroid tissue were treated with the 5 sera giving positive tanned red cell tests (see table) there was fixation of globulin by the colloid (Fig. 5). Since colloid staining was never found following treatment with normal serum, and since it was not observed when positive sera were absorbed with thyroglobulin, it appears to represent specific staining indicative of antibody to thyroglobulin. The serum (E.J.) which had no thyroglobulin antibody, failed to stain the colloid. In the one case (C.H.) where the patient's serum could be tested against his own gland, both cytoplasmic and colloid staining were obtained, confirming previous observations that true auto-antibodies are implicated in chronic thyroiditis.

The treatment with alcohol, however, modified the appearances in two ways. First, it imparted a pale blue autofluorescence to the colloid of the unstained section, so that when specific globulin fixation was followed by treatment with antiglobulin conjugate the resulting fluorescence was a darker green than is usually seen with fluorescein-labelled antisera. The second modification was the appearance of a coarse network replacing the relatively homogeneous colloid in some of the follicles (fig. 6). This was probably an artefact but nevertheless stained unequivocally with sera positive for anti-thyroglobulin.

The brightness of the colloid fluorescence in positive preparations showed considerable variation between follicles in the same section. This feature was observed with both normal and toxic glands, and with all positive sera. Of the two toxic glands examined, one gave generally brighter and more satisfactory colloid staining than the other. The colloid staining always appeared to be confined to the contents of the follicular lumen, and did not involve the adjacent epithelial cells.

Species specificity

Specific staining of both colloid and epithelium was obtained using monkey thyroids treated with positive human sera. The appearances were essentially the same as with human thyroid although the cellular staining was somewhat masked by the presence of brilliant orange auto-fluorescent granules in the cytoplasm of the monkey cells. Rabbit thyroid sections pretreated with normal guinea-pig serum showed weak cytoplasmic and colloid staining with the same

EXPLANATION OF PLATE

FIG. 1.—Unfixed section of toxic thyroid gland treated with serum L.M. containing both precipitating and complement fixing antibodies, showing uptake of specific antibody by follicular epithelium. The follicles are empty and therefore show no colloid staining.

FIG. 2.—Same as Fig. 1, but treated with serum L.M. previously absorbed with thyroglobulin. Staining of epithelial cytoplasm unaffected.

FIG. 3.—Unfixed section of normal infant's thyroid treated with unabsorbed serum L.M., showing cytoplasmic staining of follicular epithelium.

FIG. 4.—Unfixed section of toxic gland treated with normal serum. No uptake of globulin revealed by antiglobulin fluorescent conjugate.

FIG. 5.—Alcohol-fixed section of normal infant's thyroid treated with unabsorbed serum L.M. Specific staining of colloid; cellular staining abolished by prior alcohol treatment. (Compare with Fig. 3.)

FIG. 6.—A different field from the same preparation as Fig. 5, showing "network" appearance of colloid in some of the follicles.

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human sera. Somewhat similar results were briefly reported by Beutner, Witebsky and Gerbasi (1959) who found that the staining obtained using human thyroiditis sera was weaker with rabbit and dog colloid than with human gland.

These results may be compared with previous studies on the species specificity of the two thyroid auto-immune systems (Roitt, Campbell and Doniach, 1958; Roitt and Doniach, 1958); significant cross-reactions in the precipitin and complement fixation tests with Hashimoto sera were confined to monkey and chimpanzee thyroid extracts.

DISCUSSION

The results reported here clearly reveal the localization of the thyroid complement-fixing antigen in the cytoplasm of the epithelial cells and emphasize its distinctness from thyroglobulin which is confined to the colloid.

Previous studies on cytoplasmic staining of the acinar cells employing auto-antisera in the fluorescent antibody technique have been inconclusive in general and have not been interpreted in relation to the complement-fixing microsomal immune system. In White's early studies (1957) in addition to colloid staining, antigen was found as scattered round globules within the epithelial cells and in one instance, faint cytoplasmic fluorescence was noted. Hiramoto, Engel and Pressman (1958) concluded that the cytoplasmic staining they observed with chronic thyroiditis serum was not appreciably greater than that obtained with a normal serum. However, Landing, West and Esselborn (1958) found enhanced cytoplasmic staining with two out of three chronic thyroiditis sera using unfixed thyroid sections. Treatment of rabbit thyroid sections with homologous thyroid auto-antisera led to staining of the colloid which was reported as extending into the margin of the adjoining epithelium (Beutner, Witebsky, Rose and Gerbasi, 1958); the possible presence in these animal sera of auto-antibodies to the thyroid microsome fraction remains an open question, however, since the tissues were fixed in formalin which might lead to inactivation of the microsomal antigen.

In a recent study of the sera of 106 Hashimoto patients (Roitt and Doniach, 1958), the majority were shown to have antibodies directed against both thyroglobulin and the microsomal antigen; 8 sera reacted only with thyroglobulin, a further 8 gave only the complement fixation reaction and 2 failed to react with either antigen. The pathogenic significance of complement-fixing auto-antibodies directed against an intracellular antigen of the thyroid epithelium cannot yet be evaluated but it may be noted that thyroid lesions could be obtained in rabbits (Witebsky *et al.*, 1957) using purified preparations of homologous thyroglobulin which were almost certainly free of particulate subcellular components.

SUMMARY

Sections of human thyroid were treated with chronic thyroiditis sera and fixation of globulin demonstrated by application of fluorescein-conjugated anti-human globulin serum, using the technique of Coons and Kaplan.

Thyroiditis sera containing thyroglobulin precipitins gave rise to staining of the follicular colloid in alcohol-fixed sections; this staining could be prevented by prior absorption of the sera with human thyroglobulin.

Staining of the cytoplasm of acinar cells in unfixed sections was observed using patients' sera capable of reacting with the complement-fixing thyroid " micro-

somal " antigen. Cytoplasmic staining could be abolished either by absorption of the serum with a thyroid microsome preparation or by pretreatment of the thyroid section with alcohol. This staining was unaffected by absorption of the serum with thyroglobulin or with a human liver microsome preparation. The staining was organ specific. Strong cross-reactions were obtained with monkey thyroid while rabbit thyroid gave only weak staining.

The results show that the auto-antigen present in the " microsome " fraction of thyroid homogenates is derived from the cytoplasm of the epithelial cells and emphasize the distinctness of this antigen from thyroglobulin which is confined to the colloid.

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THE CORRELATION BETWEEN BONE MARROW ACTIVITY AND BLOOD NEUTROPHIL LEVELS FROM QUANTITATIVE STUDIES IN IRRADIATED GUINEA-PIGS

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THE relation between granulopoietic activity in the bone marrow and reflection of this activity in the peripheral blood is of considerable clinical significance. When haemopoietic tissues have been exposed to injury, *e.g.* irradiation, and there is the possibility of serious marrow damage, it is important to know the interval between changes in the bone marrow and their reflection in the peripheral blood. Further, in assessing recovery from granulopoenic conditions or the treatment of aplastic and leukaemic states by bone marrow replacement, it is important to know the interval between division and maturation of marrow cells and their eventual appearance in the blood.

This paper reports temporal and quantitative features of variations in marrow granulopoiesis after total body irradiation, and the effect of these variations on blood neutrophil levels. The findings are based upon quantitative studies of blood and bone marrow cells in guinea-pigs at close intervals during recovery from a single dose of whole body irradiation. In the present experiments 2 phases of increased granulopoiesis, the first of which was abortive, were observed during the recovery period. The early neutrophil leucocytosis and the abortive granulocyte recovery which followed irradiation in the present experiments are reviewed in the light of the quantitative findings. Observations are also made upon the life-spans of neutrophils and their precursors in guinea-pig bone marrow.

MATERIALS AND METHODS

Young male guinea-pigs of the Dunklin-Hartley strain and approximately 400 g. weight were given a single dose of 150–170 r whole body irradiation from a ^{60}Co unit. To ensure uniform dosage, irradiation was performed under general anaesthesia by intra-peritoneal injection of sodium pentobarbitone. The animal was supported in a wooden cage with a minimal frame and covered by a wide-meshed string net.

Using peripheral blood from an ear vein, a total white cell count and a differential count of 400 cells in at least 2 different smears were made about 7 days before irradiation. A similar investigation was made at a certain interval after irradiation, depending upon the experimental group in which the animal was placed. This was immediately followed by quantitative examination of the bone marrow using the technique described by Yoffey (1956). The quantitative technique includes differential cell counts upon bone marrow smears. At least 1,000 cells were counted in each experiment, not more than 500 cells being counted in any one smear. Samples of femoral bone marrow were examined histologically. The marrow was fixed in 10 per cent Zenker-formol, embedded in ester-wax and sectioned at 3 μ. Sections were stained with haematoxylin and eosin azure II and with toluidin blue, eosin W and orange G.

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Blood and marrow examinations were made at the following times after irradiation 4 hr., daily until the 4th day, and then on alternate days until 4 weeks after irradiation. Final observations were made 5 weeks after irradiation when haemopoietic recovery appeared to be complete. Eight animals were examined for each observation period and the results are based upon studies of a total of 144 animals. Control data were obtained from 18 animals subjected to the same experimental conditions except for actual irradiation.

Analysis of data.—The absolute numbers of neutrophils and their precursors per c.mm. marrow were combined into 3 groups. The groups are: immature cells, capable of mitosis and differentiation (myeloblasts, promyelocytes and myelocytes); intermediate forms, which undergo further maturation but do not divide (metamyelocytes and band cells), and mature forms (segmented neutrophils). The grouping is based upon observations on cell maturity by Groat (1933), Watanabe (1955) and Hayhoe and Quaglino (1958). The means and standard errors were calculated for each of the 3 groups in all the observation periods and also for the blood neutrophil levels (Table I).

RESULTS

The dose of irradiation used in the present experiments caused a mortality rate of 6·5 per cent and there were marked changes in the marrow and blood neutrophil levels in animals surviving the irradiation. Rosenthal (1955) found a mortality rate of 10–20 per cent within 30 days of 200 r whole body irradiation in guinea-pigs of 350–500 g. weight.

Changes in blood neutrophil levels

There was a marked but transient neutrophil leucocytosis 4 hr. after irradiation. By 24 hr. levels had returned to within normal limits and remained only just below normal as far as the 3rd day. The numbers then fell until the 8th day when they were less than 30 per cent of the controls. An abortive recovery now followed. Thus although levels were within normal limits on the 12th day, there was a dramatic fall in the ensuing 2 days so that on the 14th day they were only 13 per cent of control figures. Lowest levels were recorded on the 16th day when they were 6 per cent of normal. Recovery followed but was initially slow up to the 20th day when the numbers were still only about 20 per cent of normal. Subsequent to the 20th day, recovery was very rapid, levels being within control limits 2 days later on the 22nd day. Peak levels were reached 2 days later still, on the 24th day, when they were just over twice those of controls. The count then decreased to lie within normal limits on the 28th and 35th days after irradiation.

Changes in marrow cell levels

Myeloblasts, promyelocytes and myelocytes.—The levels of these cells had fallen to less than 70 per cent of the controls by 4 hr. after irradiation and the decrease continued until the 3rd day when they were only approximately 20 per cent of the controls. In the temporary recovery which followed, maximum numbers, about 80 per cent of normal, were found on the 6th day after irradiation. A steady decrease then followed and minimum levels were noted on the 12th day, being 14 per cent of control values. After the 12th day there was definitive recovery and the numbers gradually increased until the 20th day when they were 70 per cent greater than normal. A return to normal levels followed the peak of recovery and they remained within almost normal limits from the 24th day onwards.

TABLE I.—*Quantitative Changes in Marrow Neutrophils and their Precursors, and in Blood Neutrophils of Guinea-pigs after Whole Body Irradiation*

Time after irradiation	Marrow cells (per c.mm. marrow ± standard error)			Blood neutrophils (per c.mm. blood ± standard error)
	Myeloblast, Promyelocyte and N. Myelocyte	N. Metamyelocyte and N. Band cell	Segmented neutrophil	
Controls	110,000 ±4,000	370,000 ±12,000	173,000 ±11,000	1,820 ±250
4 hr.	75,000 ±5,000	318,000 ±27,000	98,000 ±12,000	7,890 ±1,810
1 day	51,000 ±4,000	369,000 ±23,000	269,000 ±17,000	1,300 ±310
2 days	27,000 ±6,000	154,000 ±12,000	224,000 ±15,000	1,090 ±210
3 ,,	25,000 ±3,000	129,000 ±8,000	243,000 ±31,000	1,180 ±150
4 ,,	38,000 ±6,000	94,000 ±23,000	210,000 ±53,000	860 ±180
6 ,,	90,000 ±13,000	123,000 ±21,000	77,000 ±19,000	620 ±160
8 ,,	43,000 ±6,000	284,000 ±26,000	107,000 ±26,000	500 ±100
10 ,,	18,000 ±4,000	228,000 ±56,000	191,000 ±34,000	780 ±150
12 ,,	16,000 ±3,000	25,000 ±6,000	135,000 ±16,000	1,670 ±150
14 ,,	34,000 ±9,000	2,000 ±700	4,000 ±2,000	240 ±60
16 ,,	82,000 ±13,000	36,000 ±15,000	11,000 ±6,000	110 ±40
18 ,,	184,000 ±31,000	269,000 ±94,000	31,000 ±10,000	160 ±80
20 ,,	189,000 ±8,000	379,000 ±46,000	110,000 ±28,000	360 ±130
22 ,,	135,000 ±16,000	654,000 ±67,000	344,000 ±24,000	1,980 ±540
24 ,,	107,000 ±7,000	488,000 ±64,000	404,000 ±60,000	3,750 ±1,080
26 ,,	89,000 ±10,000	410,000 ±23,000	261,000 ±45,000	2,380 ±590
28 ,,	86,000 ±15,000	336,000 ±39,000	141,000 ±14,000	1,550 ±220
35 ,,	89,000 ±8,000	307,000 ±29,000	104,000 ±13,000	1,480 ±260

Metamyelocytes and band forms.—The levels fell slightly 4 hr. after irradiation but were within normal limits by 24 hr. The numbers then decreased until the 4th day when they were 25 per cent of normal. As in the more immature cells, a temporary recovery followed, the numbers increasing until the 8th day when they were just under 80 per cent of normal. In the decrease which followed the abortive recovery, the fall was especially rapid after the 10th day and in the following 48 hr. there was a dramatic reduction in numbers. By the 14th day these cells were virtually absent from the marrow, their levels being less than 1 per cent

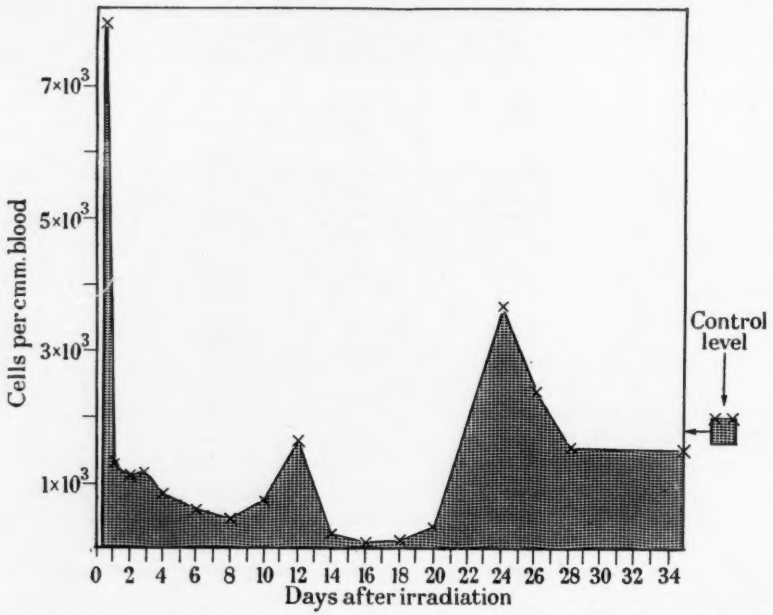


FIG. 1.—Changes in blood neutrophil levels of guinea-pigs after whole body irradiation.

of the controls. In the final recovery, these cells increased very rapidly in the 48 hr. subsequent to the 16th day and reached peak levels by the 22nd day when they exceeded the control figures by more than 75 per cent. The levels then fell and were slightly subnormal on the 35th day after irradiation.

Segmented neutrophils.—Concomitantly with the neutrophil leucocytosis 4 hr. after irradiation, the levels of segmented neutrophils in the marrow fell significantly to 56 per cent of normal. The numbers then returned to levels slightly greater than normal as far as the 4th day but rapidly decreased in the subsequent 2 days. Thus on the 6th day they were only 44 per cent of control levels. There followed, as in the more immature cells, 2 recovery peaks. The first peak was abortive but the second proved to be definitive. In the abortive recovery, maximum levels were recorded on the 10th day when they were within control limits. However, between the 12th and 14th days the numbers fell rapidly and by the

14th day segmented neutrophils were almost absent from the marrow, being only about 2 per cent of normal values. For the next 4 days the levels of segmented cells remained extremely low but there was marked recovery after the 18th day. The recovery was particularly rapid in the 48 hr. between the 20th and 22nd days after irradiation. Peak levels were reached on the 24th day when they were more than 130 per cent above normal. At this time the marrow, macroscopically,

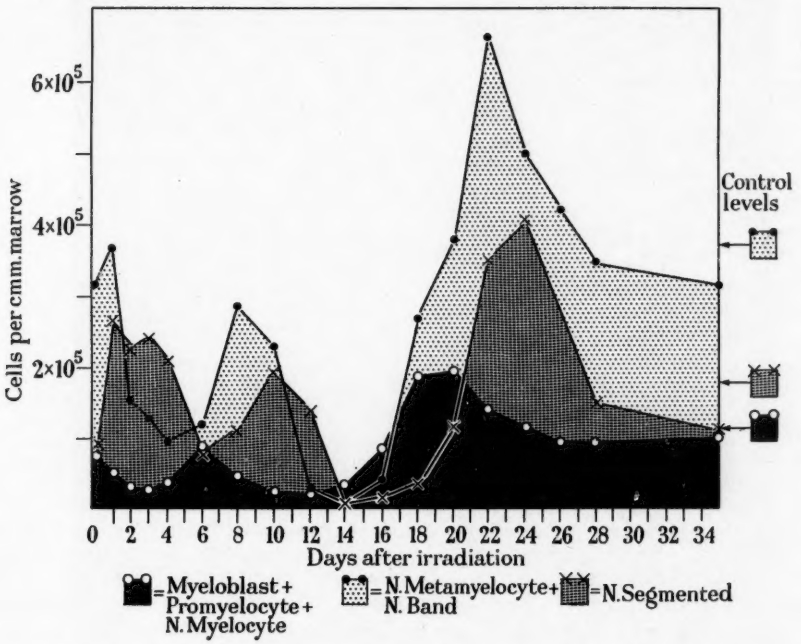

6×10⁵
4×10⁵
2×10⁵
Cells per cmm. marrow
Control levels
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Days after irradiation
=Myeloblast + Promyelocyte + N. Myelocyte
=N. Metamyelocyte + N. Band
=N. Segmented

FIG. 2.—Changes in levels of neutrophils and their precursors in guinea-pig bone marrow after whole body irradiation.

had a typical pale salmon-pink colour as in myeloid leukaemia and microscopically marrow sections showed large numbers of mature neutrophils (Fig. 3). The number of segmented cells decreased after the 24th day to reach slightly subnormal levels on the 28th and 35th days.

Time Relations Between Changes in Marrow Neutrophil Populations and Blood Neutrophil Levels

Comparison between increased activity in marrow cells and its reflection in the peripheral blood

It has been noted that there were 2 periods of increased activity following irradiation. The first was abortive but the second proved to be final recovery. In both periods the increase in cells was in a definite sequence, occurring first

in the most immature cells of the marrow, then in the more differentiated cells and finally in the blood.

TABLE II.—*Time Relations Between Changes in Marrow Neutrophil Populations and in Blood Neutrophil Levels*

Increased activity

Cells	Occurrence of 1st peak	Interval	Occurrence of 2nd peak	Interval
Marrow cells—				
Myeloblast, Promyelocyte and N. Myelocyte	6th day	2 days	20th day	2 days
N. Metamyelocyte and N. Band cell	8th day	2 days	22nd day	2 days
Segmented neutropil	10th day		24th day	
Blood cells—		2 days		0
N. polymorph	12th day		24th day	
Time from peak in neutrophil precursors to blood peak		6 days		4 days

Decreased activity

Time after irradiation	Decrease in neutrophil precursors	Decrease in blood cells	Interval for reflection of marrow decrease in blood
Immediately following irradiation	Within 4 hr.	4th day	4 days
Following 1st peak	8th day	14th day	6 days
Following 2nd peak	22nd day	26th day	4 days

Abortive recovery.—The peak in the most immature marrow cells occurred on the 6th day after irradiation and that in the more mature cells, i.e. metamyelocytes and band forms, followed 2 days later, on the 8th day. The peak in the segmented neutrophils of the marrow was on the 10th day after irradiation and occurred 4 days after that in the immature cells. Maximum levels in blood neutrophils followed 6 days after those in the immature marrow cells, i.e. on the 12th day.

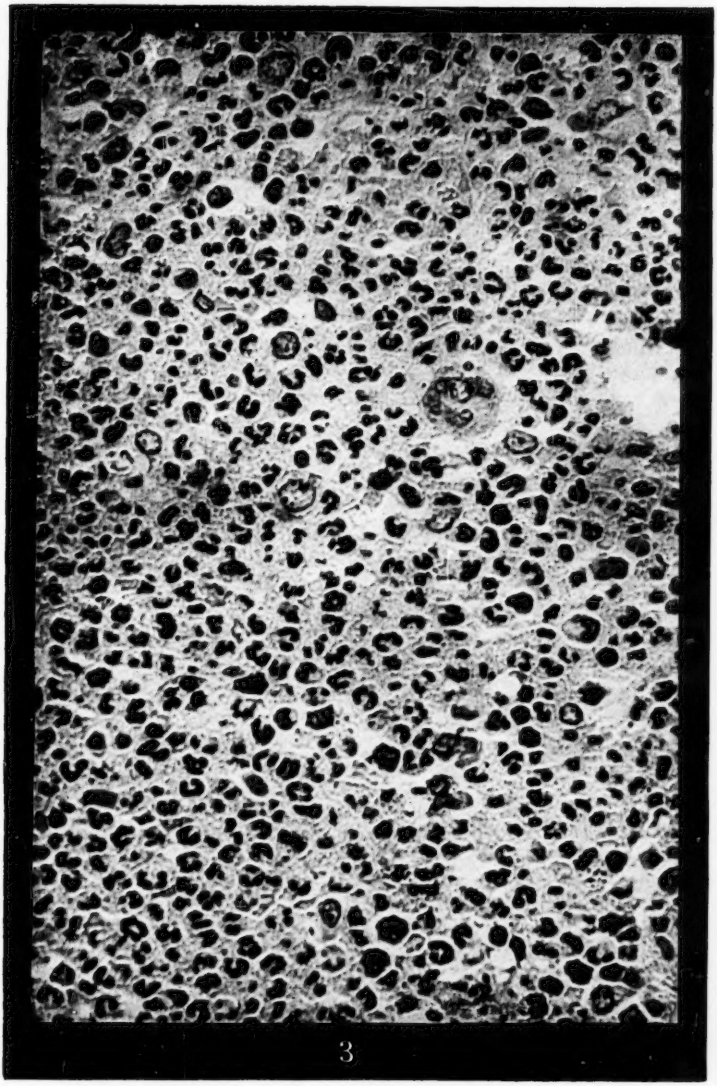
Final recovery.—The highest levels in the immature marrow cells were found on the 20th day after irradiation. The peak in metamyelocytes and band forms occurred 2 days later on the 22nd day, and that of the segmented cells was 4 days after the peak in immature cells. Maximum levels in blood neutrophils were noted on the 24th day and coincided with the peak levels of segmented cells in the marrow.

Comparison between decreased activity in marrow cells and its reflection in the peripheral blood

Despite the marked discharge of segmented cells from the marrow 4 hr. after irradiation, which coincided with a neutrophil leucocytosis in the blood, the blood

EXPLANATION OF PLATE.

FIG. 3.—Guinea-pig bone marrow 22 days after irradiation, showing many mature neutrophils. Haematoxylin and eosin azure II. × 500.



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neutrophil levels remained only just below normal up to 3 days after irradiation. The immature marrow cells decreased progressively from the time of irradiation. Thus 4 days elapsed before decreased marrow activity was definitely reflected in the blood neutrophil levels. Following the abortive recovery in the marrow, 6 days elapsed between a decrease in immature marrow cells and a fall in blood neutrophils. In the final recovery, there was an interval of 4 days between the decrease in immature marrow cells and the fall in blood neutrophils.

TABLE III.—*Ratios of the Three Groups of Neutrophils and their Precursors in Guinea-pig Bone Marrow after Whole Body Irradiation*

	Myeloblast, Promyelocyte and N. Myelocyte		N. Metamyelocyte and N. Band cell		Segmented neutrophil
1st peak (abortive recovery)	1	:	3·2	:	2·1
2nd peak (final recovery)	1	:	3·4	:	2·1
Controls	1	:	3·3	:	1·6

Ratios of the three groups in marrow neutrophils and their precursors

Since the peak levels in the 3 groups of marrow cells represent comparable points, the ratios of the 3 groups at the times of maximum activity during the abortive and the final recovery were compared. These ratios were found to be similar and could be compared with those in control animals. In calculating the ratios, the immature cells i.e. myeloblasts, promyelocytes and myelocytes were taken as unity.

Ratio of blood neutrophils to segmented neutrophils in bone marrow

Comparisons were made between the peak levels of segmented cells in the marrow and the peak levels of neutrophils in the blood. The comparison was also made using data from control animals. In both the experimental and control animals there is a fairly constant ratio between blood neutrophils and the segmented neutrophils in the marrow (Table IV). Thus at the time of the abortive

TABLE IV.—*Ratios Between Blood Neutrophils and Segmented Neutrophils in Guinea-pig Bone Marrow after Whole Body Irradiation*

	1st peak	2nd peak	Controls
Marrow (cells per c.mm.)	191,000	404,000	173,000
Blood (cells per c.mm.)	1,670	3,750	1,820
Ratio	1 : 114	1 : 108	1 : 95

peak, for every 114 segmented neutrophils per c.mm. marrow, 1 neutrophil ultimately appeared in the peripheral blood. In the final recovery peak, for every 108 segmented neutrophils per c.mm. marrow, 1 neutrophil eventually appeared in the blood. In control animals the ratio between blood neutrophils and segmented marrow neutrophils was 1 to 95.

Quantitative comparison of the magnitude of the abortive and final recovery

The quantitative data enable direct comparison of the size of the abortive recovery and the final recovery. Table V shows that in all 3 groups of marrow

cells and in the resulting leucocytosis, the final recovery was approximately twice as great when compared with the abortive recovery.

TABLE V.—*Quantitative Comparison of the Abortive and Final Recovery in Marrow and Blood Neutrophils in Guinea-pigs after Whole Body Irradiation*

Cells	1st peak (cells per c.mm. marrow or blood)	2nd peak (cells per c.mm. marrow or blood)
Myeloblast, Promyelocyte and N. Myelocyte	90,000	189,000 = × 2·1
N. Metamylocyte and N. Band cell	284,000	654,000 = × 2·3
Segmented neutrophil	191,000	404,000 = × 2·1
Blood neutrophils	1,670	3,750 = × 2·2

DISCUSSION

The Interval Between Changes in Marrow Granulopoiesis and Their Reflection in the Peripheral Blood

Lawrence, Ervin and Wetrich (1945) and Osgood (1954) suggest that blood neutrophils represent a transient and even a recurring phase as neutrophils pass between extravascular sites. The blood neutrophil levels will be determined by the difference between the rates at which these cells enter and leave the circulation. How significant are blood neutrophil levels during disturbances in marrow activity? In the present experiments variations in granulopoiesis which followed whole body irradiation were eventually reflected in the blood. But it was also found that in the early stages of marrow damage, when reserves of segmented cells still remain in the marrow, a considerable leucocytosis can occur despite a decline in immature cells. The changes in blood neutrophil levels which follow irradiation, once established, may be drastic and occur relatively rapidly. Thus, between the 12th and 14th days after irradiation a marked fall in levels occurred within 48 hr. However, recovery may occur with equal rapidity and there was a dramatic increase in levels between the 20th and 22nd days. Cronkite and Brecher (1955) suggested that after irradiation a critical level in blood granulocytes may be reached, below which survival is improbable. The present findings suggest that recovery may still occur despite a marked neutropoenia and its attendant dangers. Thus, on the 16th day after irradiation the blood neutrophil levels were only 6 per cent of the controls but they had returned to normal by the 22nd day, most of the recovery occurring within 48 hr.

In the present experiments the interval between increased activity in precursors of marrow neutrophils and the eventual rise in blood neutrophils was 6 days in the abortive recovery and 4 days in the final recovery. The quantitative data show a prolonged retention of segmented cells in the marrow during the abortive recovery. Elson, Galton and Till (1958) found hypersegmented neutrophils in the marrow of rats after injection of Chlorambucil and the discharge of these cells into the blood was delayed. In the present experiments hypersegmented neutrophils were noted in blood smears 12 days after irradiation. This supports the prolonged retention of segmented cells in the marrow during the abortive recovery, maturation proceeding during their retention. The neutropoenia preceding the abortive recovery was less severe than that before the final recovery. If blood levels reflect the general neutrophil levels in the body, then greater

numbers existed before the abortive recovery and consequently the demand for their discharge from the marrow might be less urgent.

In the guinea-pig, the interval of 4 days found in the final recovery appears to represent the recovery time for blood neutrophil levels after relatively severe marrow damage. The marked marrow depletion in the present experiments may have increased mitotic rates and hastened maturation when compared with normal conditions. Rosenthal (1955) illustrates an interval of 4 days between peak levels of myelocytes and segmented neutrophils in the marrow of young guinea-pigs recovering from 200 r irradiation. Maximum levels of segmented cells in the marrow coincided with a neutrophil peak in the blood, as in the present experiments. Craddock, Perry and Lawrence (1956) found a delay of 3–4 days before labelled cells were released into the blood of dogs whose marrow reserves of segmented cells had been previously exhausted. Depletion of reserves appeared to hasten maturation since the corresponding interval in normal dogs was 4–5 days. Osgood (1954) found the interval between formation of myelocytes and segmented neutrophils in cultures of human marrow to be about 4·5 days. Kline and Cliffton (1952), from studies with ^{32}P, concluded that human leucocytes remained in the bone marrow for 4 days. Clinical evidence in humans suggests an interval of approximately 4–5 days before marrow regeneration is shown in the peripheral blood (Bernhard, Echikson, Grubin and Zins, 1956 ; Schick and Virks, 1956). Bone marrow transplantation experiments provide some information on the relation between marrow activity and blood leucocyte levels. Nowell, Cole, Roan and Habermeyer (1957) found increasing numbers of rat leucocytes in the blood and bone marrow of severely irradiated mice from the 5th day after intravenous injection of rat marrow. Porter (1957) noted a rapid rise of female heterophils in the blood of irradiated male rabbits 3–5 days after injection of marrow from female donors.

Data from the present experiments suggest an interval, in the guinea-pig, of about 4 days between a decrease in neutrophil precursors and reflection of this in the peripheral blood. After the abortive recovery the interval was 6 days, and this appears to be due to the prolonged retention of segmented neutrophils in the marrow at this time. Lawrence *et al.* (1945) found a marked leucopoenia in cats 3–5 days after severe whole body irradiation.

Life Span of Neutrophils and Their Precursors in Guinea-pig Bone Marrow

Findings in the present experiments suggest that the life-span of the various forms of marrow neutrophil increases as the cell matures. The peak levels of myeloblasts, promyelocytes and myelocytes coincided in both the abortive and final recovery. Thus mitosis and differentiation of myeloblasts into promyelocytes and of the latter into myelocytes appear to occur within 24 hr. Differentiation of myelocytes through the next 2 stages, i.e. metamyelocyte and band form, lasts 48 hr. The final maturation from band form into segmented cell occupies a further 48 hr.

In the abortive granulopoietic recovery described by Rosenthal (1955), the peak levels of myeloblasts, promyelocytes and myelocytes coincided on the 6th day, as in the present experiments. Osgood (1954) described an increase in life span as cells matured in cultures of human marrow. The duration of myelocytes and metamyelocytes was estimated to be 24 hr., of band forms—36 hr. and of segmented cells 48 hr. Lajtha (1957) observed that in vitro experiments indicate

that late forms of normoblast have a longer lifetime in marrow than early forms. Lajtha (1959) noted an interval of about 48 hr. for the formation of band and segmented cells from labelled promyelocytes and myelocytes in cultures of human marrow.

Quantitative findings in the marrow of control animals show that the number of immature cells is small when compared with that of the more mature cells. The simile of a leucon " pyramid " may be used to describe this characteristic cell distribution, the most immature cells forming the apex and the much larger number of more mature cells forming the base. In the present experiments, the finding of an increased life span in marrow granulocytes, as they mature, suggests an explanation for their characteristic quantitative distribution in normal marrow. Thus, the more immature cells, having a shorter life span when compared with mature forms, will occur less frequently in any particular marrow sample.

The Initial Leucocytosis Following Whole Body Irradiation

There was a marked neutrophil leucocytosis 4 hr. after irradiation in the present experiments. Jacobson (1954) has suggested 2 factors in the production of the irradiation leucocytosis: hastened maturation of granulocyte precursors (see Isaacs, 1932), and mobilization of cells following widespread tissue injury.

In the present experiments the decrease in metamyelocytes and band cells 4 hr. after irradiation was comparatively small. Craddock *et al.* (1956) and Yoffey (1956) stress the substantial reserves of mature neutrophils in active bone marrow. The present experiments show that, quantitatively, the leucocytosis following irradiation could entirely result from mobilization of segmented cells from the marrow reserves. Knowing the active marrow volume in a 400 g. guinea-pig (Hudson, 1958) and also its blood volume (Ancill, 1956), and using the quantitative data from the present experiments, then the absolute decrease in marrow neutrophils 4 hr. after irradiation and the absolute number of blood neutrophils at this time can be calculated. These calculations show that approximately 468×10^6 segmented neutrophils had been discharged from the marrow 4 hr. after irradiation. The total number of neutrophils present in the circulation at this time was approximately 227×10^6. Thus the blood neutrophils account for only about 50 per cent of the segmented cells discharged from the marrow. Harris, Menkin and Yoffey (1956) noted a similar discrepancy after the injection of a leucocytosis promoting factor into guinea-pigs. The " missing " neutrophils could be accounted for in extravascular compartments, where Osgood (1954) has estimated that large numbers occur, or by sequestration in viscera (Bierman, Kelly and Cordes, 1955).

In the present experiments, only 20 hr. after the considerable discharge of segmented cells from the marrow, their numbers there had recovered to levels even greater than normal. The neutrophil leucocytosis had ceased by this time. The magnitude and speed of this recovery suggest that the levels could not be restored merely by maturation of precursor cells but involved a redistribution of neutrophils by the end of the leucocytosis. The observation of Osgood (1954) that leucocytes are a motile group, constantly moving to sites where they are most needed, supports this concept. Further, Rogers and Melly (1957), studying bacteraemia in rabbits, found that after temporary sequestration of granulocytes, some appeared to return into the circulation. Gowans (1958) has demonstrated

the continuous recirculation of lymphocytes through the blood stream. A similar recirculation of neutrophils may reasonably be postulated.

The Abortive Recovery Following Whole Body Irradiation

Abortive marrow recovery has been noted between the 4th and 11th days after whole body irradiation in several species (Jacobson, 1954). In the present experiments, an abortive recovery in neutrophils occurred between the 4th and 12th days after irradiation. The quantitative data show that the magnitude of this recovery in all its phases, including the resulting leucocytosis, was almost half as great as in the final recovery. Further, the ratios between the peak levels of granulocytes in the various marrow subgroups in both the abortive and the final recovery were very close and could be compared with those in controls. Also, during both recovery phases, the ratios between the ultimate levels of segmented cells in the marrow and neutrophils appearing in the blood were very close and similar to the control ratio. The similarity of these ratios suggests an orderly recovery pattern from the irradiation used in these experiments, the number of precursor cells in the marrow providing a fairly accurate indication of the ultimate levels of segmented neutrophils in the marrow and blood which may be expected. Jacobson (1954) observed that the abortive recovery might be due to the multiplication of cells which were injured at the time of irradiation but which died after a limited number of divisions. Elson *et al.* (1958) have given an interesting interpretation of an abortive recovery in rat erythroid cells after administration of Busulphan. Two stages are postulated in erythropoietic cells. Cells in the 1st stage are more primitive and have a longer intermitotic interval than those in the 2nd stage. Assuming that Busulphan affects the intermitotic times in both stages proportionately, then the abortive peak was suggested to represent resumed proliferation in only 2nd stage cells. In the present experiments, on the 6th day after irradiation, a definite peak in myeloblast levels initiated the abortive recovery in neutrophils. Myeloblasts in the granulocytic series would correspond to the 1st stage erythroid cells postulated by Elson and co-workers.

SUMMARY

Young male guinea-pigs were given 150–170 r whole body gamma irradiation from a ^{60}Co unit. Quantitative studies were made upon neutrophils and their precursors in bone marrow and upon blood neutrophil levels at frequent intervals up to the 5th week after irradiation, when haemopoietic recovery appeared to be complete. The damage produced by the irradiation was followed by 2 recovery phases in the marrow, both of which were eventually reflected in the peripheral blood. The 1st phase occurred between the 4th and 12th days after irradiation and was abortive. The 2nd phase commenced about the 14th day and led to complete recovery. The quantitative data revealed an orderly recovery pattern in marrow neutrophils and their precursors in both phases of recovery.

The abortive recovery was shown by all stages of marrow neutrophils and was initiated by an increase in myeloblasts. In all its phases this recovery was, quantitatively, about half as great as the final recovery. There was prolonged retention of segmented neutrophils in the marrow during this period.

These experiments show that, once established, the changes in blood neutrophil levels which reflect variations in marrow activity may occur rapidly with marked

rises or falls in levels. Recovery may still occur despite a marked neutropoenia. In guinea-pigs, after severe marrow damage, the interval between increased activity in early neutrophil precursors and reflection of this in the peripheral blood is approximately 4 days. There is a similar interval between decreased activity in stem cells of neutrophils in the marrow and reflection of this activity in the blood.

The quantitative findings show that the leucocytosis which rapidly follows irradiation can be entirely explained by the discharge of segmented neutrophils from the marrow reserves.

These experiments suggest that neutrophils in the marrow have an increasing life span as their maturation proceeds. This variation in life span may explain the characteristic quantitative distribution of the various stages of granulocyte in active bone marrow.

I am very grateful to Professor J. M. Yoffey for suggesting quantitative studies of haemopoiesis in irradiated animals and also for providing research facilities. I wish to thank Professor F. Davies and Dr. E. K. Blackburn for helpful advice in the preparation of this paper. I also thank Dr. R. C. Tudway for irradiation facilities in the Radiotherapy Department, Bristol General Hospital and the hospital physicists for their constant help.

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THE PRODUCTION OF FATTY LESIONS IN THE PULMONARY ARTERIES OF RABBITS BY THE INTRAVENOUS INJECTION OF THROMBI

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THE original object of these experiments was to seek evidence about the possible source of lipids in atheromatous plaques. The hypothesis considered was that the fat might be derived from erythrocytes. This possibility was suggested by the fact that envelopes of red blood cells are rich in lipid; that recurrent thrombosis and haemorrhage occur at the bases of plaques in close proximity to deposits of fat (Winternitz, 1947; Geiringer, 1951); that haemosiderin is frequently found in these areas (Paterson, Moffat and Mills, 1956); and that thrombi containing enmeshed red blood cells are deposited on the arterial intima and may be the initiating factor in atherosclerosis (Duguid, 1957). An attempt was therefore made to achieve the incorporation of thrombi with and without erythrocytes in an arterial wall with a view to comparing the fat content of the resulting lesions.

The question whether the presence of erythrocytes was necessary for the production of lesions containing fat was not answered because of failure to produce lesions in which erythrocytes were not involved. However this work did show clearly that clots containing red blood cells could be incorporated into the intima, that the resulting lesions very frequently contained a large amount of fat, and that such lesions could be produced in rabbits whose lipid metabolism was not disturbed. Despite the use of similar methods by other workers, such lesions have not been described before, though since this work was completed similar lesions have been described in veins by Filshie and Scott (1958).

METHODS AND MATERIALS

Lesions in the pulmonary arteries were produced by injecting clots into the ear veins of male mongrel rabbits.

Rabbits always received injections of clots prepared from their own blood. To prepare clots containing erythrocytes, 1 ml. of blood was withdrawn, allowed to clot in a beaker and then gently fragmented in a "Tosco" homogenizer with the blades rotating slowly for 10 min. This consistently produced clots of roughly cubical shape whose dimensions ranged from 0·25–1·0 mm. The fragments were resuspended in 1 ml. saline. Injections were given to 6 rabbits at twice weekly intervals for 4, 4, 4, 5, 6 and 6 weeks respectively.

To prepare clots free from erythrocytes, 2 ml. blood was collected in siliconed tubes and centrifuged at 2000 r.p.m. for 10 min. The plasma was pipetted off and allowed to clot. The clot was fragmented as already described, and pieces of similar dimension to those containing red blood cells were injected into 3 rabbits at twice weekly intervals for 4, 4 and 5 weeks respectively.

One rabbit in each group died suddenly after the eighth injection. The other rabbits were killed with nembutal.

The main branches of the pulmonary arteries were opened and examined immediately after death. The lungs were then fixed in formalin. Frozen sections were stained with Sudan

IV, and paraffin sections were cut and stained with haematoxylin and eosin and with a Prussian blue stain.

The right ventricular pressures were successfully measured by percutaneous ventricular puncture under anaesthesia in 3 rabbits which received clots containing red blood cells and in one rabbit which received fibrin clots.

In all animals the ventricles were separated and weighed after death.

RESULTS

There was no evidence of pulmonary infarction.

In both groups of animals, when an injection had been given shortly before death, fresh clots were found lodged in the orifices of the main branches of each pulmonary artery (Fig. 1). Older clots were also found obstructing some orifices.

In 5 out of 6 rabbits into which erythrocyte containing clots were injected and in 2 out of 3 rabbits which received fibrin thrombi only, there were several yellow lesions in the main pulmonary arteries (Figs. 2 and 3). They were 0·5–1·0 mm. wide and up to 8 mm. long. They were found at the orifices of large branches but were retracted to one side of the vessel and covered by endothelium. Their position was identical to recently injected clots at other sites. A few other clots were found in which recent red thrombus was flecked with small yellow spots. Microscopically these yellow lesions in both groups were identical. They were polypoid and covered by a layer of endothelium. They consisted mainly of foam cells, some extracellular fat, fibroblastic tissue and some fibrin (Figs. 4 and 5). Staining with Sudan IV showed that a great deal of fat was present, and staining for iron revealed the presence of much haemosiderin.

In both groups in the peripheral smaller arteries different lesions were found which resembled those described by Harrison (1948) and others (Wartman, 1951 ; Barnard, 1954 ; Thomas, O'Neal and Lee 1956). Organisation of thrombi was seen at all stages. Some recent clots produced complete obstruction of the artery and older ones were retracted to one side. They were invaded by fibroblastic tissue and covered by endothelium. Small eccentrically placed lesions appeared to consist largely of new fibrous tissue. There was frequently a cellular reaction within and outside the wall of an artery. Such lesions were found in great numbers throughout the lung, but fat was seen in very few sections and then only in very small amounts.

Right ventricular pressures recorded in 4 rabbits, 3 of which had received erythrocyte containing clots and one fibrin clots, were 22/6, 22/10, 20/8 and 24/8 mm. Hg. respectively. Pressures in three normal rabbits were 24/6, 20/4 and 22/6 mm. Hg. respectively.

EXPLANATION OF PLATE

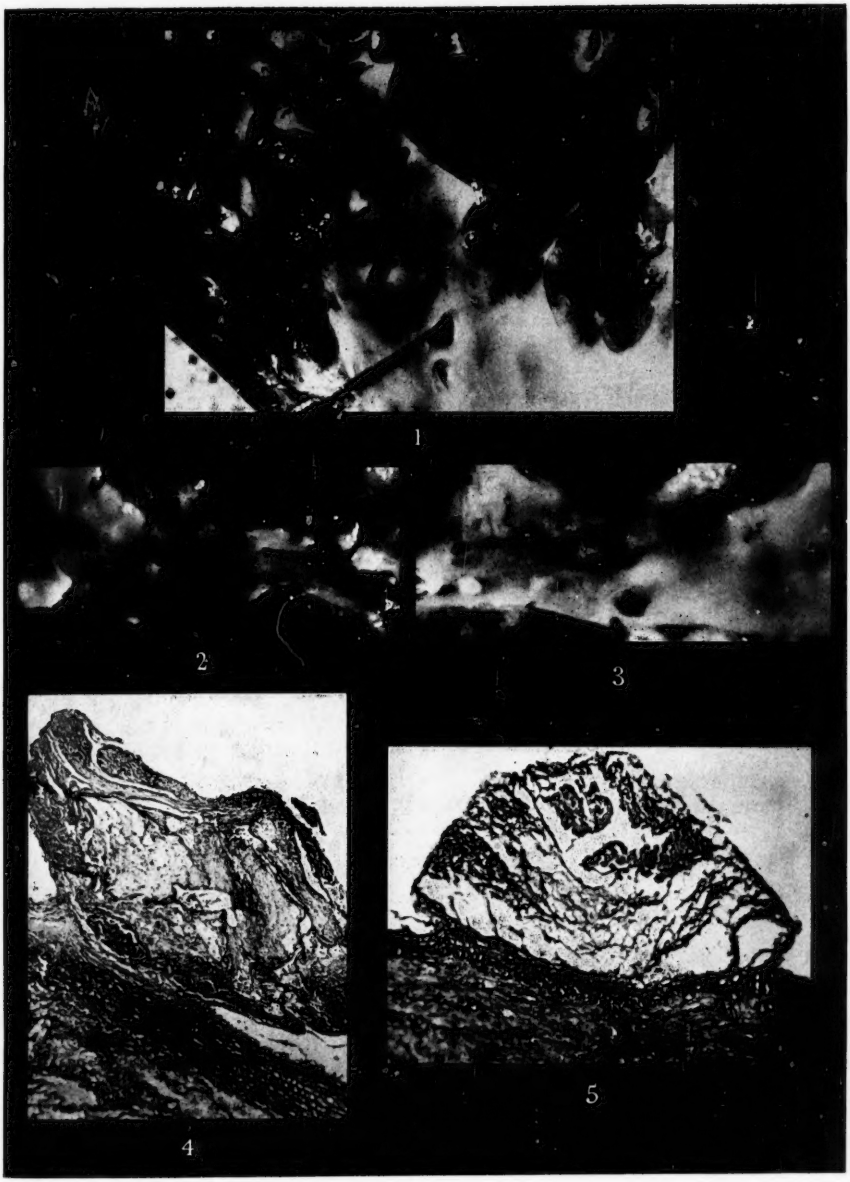
FIG. 1.—Fresh red blood cell clots in the orifices of the main branches of a pulmonary artery × 6.

FIG. 2.—Fatty lesion in a pulmonary artery. × 6.

FIG. 3.—Three lesions in a pulmonary artery : one small fatty lesion in lower left hand corner and two more recent lesions which contained fat and red clot. × 6.

FIG. 4.—Low power view of a section taken from Fig. 3 showing a clot on the surface of a pulmonary artery. Fibrin and erythrocytes are seen on the surface and foam cells towards the centre. Frozen section. Sudan IV and haematoxylin. × 75.

FIG. 5.—Low power view of a section taken from the artery in Fig. 2. A clot containing fibrin and foam cells is shown attached to the intima. Frozen section. Sudan IV and haematoxylin. × 100.



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The ratio of the weights of the right and left ventricles were calculated for all the injected animals and the same number of normal rabbits. Expressed as percentages the mean ratios for the injected animals was 30·8 per cent and for the normal animals 29·1 per cent. These means do not differ significantly.

DISCUSSION

The results show that thrombi which have become attached to the intima of a large artery may after some time become incorporated in the arterial wall and be converted into a lesion which contains much fat. The finding of considerable haemosiderin in all these fatty lesions shows that red cells were present in large numbers at some stage. In the case of the fibrin clots it indicates that secondary thrombosis must have occurred after the clots had become lodged in the orifices of branches of the pulmonary artery.

Although 7 of the 9 rabbits were affected only 2 or 3 lesions were found in each animal. The paucity of emboli which were retained in the pulmonary circulation has been the general experience of workers using this technique. Rabin, Thomas, Lee, Konikov and Scott (1957) used a transparent ear chamber to observe directly the fate of blood clots labelled with india ink which they injected into the central artery of a rabbit's ear. They found that the majority of such clots disintegrated rapidly and did not become attached to the arterial wall. It seems that the rabbit has a highly efficient system for dealing with emboli.

The lesions of the large pulmonary arteries appear hitherto to have escaped attention. They were confined to the right and left branches of the main pulmonary arteries. Wartman, Jennings and Hudson (1951), Barnard (1954), Thomas *et al.* (1956) and Heptinstall (1957), whose experimental methods were very similar to the present one, apparently did not examine the arteries in the fresh state. The lungs were fixed in formalin immediately after death and blocks were subsequently taken from the periphery. They found, as was the case in the present study, that it was exceptional for fat to be found in the more distal type of lesion which they studied. As in this study, Harrison (1948), Barnard (1954) and Thomas *et al.* (1956) found that persisting pulmonary hypertension was not produced.

The difference in the type of lesion found in the main pulmonary arteries and the smaller arteries is in itself interesting. It is possible that the fat content of the experimental lesion depends on the size of the occluding thrombus which would have been much larger in the main branches than in the periphery. Filshie and Scott (1958) investigated the fate of thrombi in the portal circulation, and found that large polypoid lesions containing fat occurred only in the larger veins where large erythrocyte containing thrombi were previously shown to have lodged. In the smaller veins only fibrin clots were encountered and no fat was found. Their work and the present study therefore strongly suggest that in arterial lesions initiated by deposition of thrombi, fat is found in large quantity only where large thrombi containing many red blood cells have been deposited. In other pathological states it is common to find fat where thrombosis or haemorrhage have previously occurred, and foam cells containing fat are also found after the subcutaneous injection of red blood cells (Muir and Niven, 1935). The possibility remains that the lipid rich envelopes of erythrocytes is the source of some of the fat in arterial lesions, but it is equally likely that fat has an affinity for degenerating red cell deposits into which it infiltrates from the plasma.

SUMMARY

The possibility that the fat in atheromatous plaques might be partly derived from erythrocytes was investigated. Thrombi with and without red blood cells were injected intravenously into two groups of rabbits and the pulmonary arteries were subsequently examined.

The incorporation of the clots into the intima of the larger pulmonary arteries frequently produced lesions which contained a large amount of fat. The lesions in both groups of rabbits were identical and in addition to fat also contained haemosiderin. In the case of those rabbits which received clots without red blood cells, the presence of haemosiderin was probably due to secondary thrombosis. The question whether the presence of erythrocytes was necessary for the production of lesions containing fat was not answered because of the failure to produce lesions in which erythrocytes were not involved. The fatty lesions were found in 7 out of 9 rabbits used, and had been produced in rabbits whose lipid metabolism had not been disturbed.

Although similar methods have been used in the past such lesions have not been described before, possibly because only the peripheral parts of the pulmonary arteries had been examined. The ratios of the weights of the two ventricles and direct right ventricular pressure readings were found to be normal.

I wish to thank Professor R. R. H. Lovell for much helpful advice and Miss E. Flower for technical assistance.

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THE D → C CHANGE IN POLIOVIRUS PARTICLES*

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POLIOVIRUS suspensions, crude or purified, of each of the 3 serological types contain particles possessing one or other of two different, type-specific, antigenic reactivities—D or C. The differentiation and measurement of the D and C antigens have been discussed in a previous paper (Le Bouvier, 1959) in which were defined the terms " D antigen " and " C antigen " as used in this study. Their meaning is qualified by the method employed, which has been that of antigen-antibody precipitation in agar. The conclusions reached apply to the specific configurations taking part in that particular reaction. These configurations are not necessarily identical with those detected by other methods, such as complement fixation or flocculation in liquid mixtures. Nevertheless, from tests done on comparable sucrose-density-gradient fractions of purified poliovirus (Schwerdt and Schaffer, 1956 ; Schaffer and Schwerdt, 1959) it appears that the D-antigenic configuration reacting by complement fixation and that reacting by precipitation are both present on the same kind of particle, whatever may be the degree of overlap between the precise chemical groups involved (Mayer, 1957 ; Mayer *et al.*, 1957 ; Le Bouvier, Schwerdt and Schaffer, 1957). The same holds good for the C-antigenic configuration.

The evidence from absorption tests indicates that, at least in the conditions so far examined, the D and C antigenic characters are associated with different particles (Hummeler and Hamparian, 1958 ; Le Bouvier, 1959). If there is a state in which the two kinds of configuration can co-exist, with full activity, on the selfsame particle, then it would appear to be an ephemeral one ; or else, if stable, one that is present in only a small proportion of the population. Results obtained by flocculation of liquid mixtures, however, suggest that the qualification " with full activity " may be important (Smith, unpublished observation).

Besides D antigen, unheated poliovirus concentrates may also contain " spontaneous " C antigen in detectable amounts. The proportions of D and C vary from one concentrate to another ; but in most fresh preparations the quantity of C antigen is relatively small and may even be undetectable in agar-diffusion tests. When such concentrates are heated, or treated in various other ways, they are changed (Roizman, Mayer and Rapp, 1958 ; Hummeler and Hamparian, 1958 ; Roizman, Mayer and Roane, 1959). Having previously been largely or entirely D, the specific reactivity now becomes indistinguishable, by all immunological tests so far applied, from that of the " spontaneous " C antigen present in unheated crude virus preparations or in the purified sucrose-density-gradient fraction SDG^C. This alteration of character is accordingly referred to as the

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D → C change. The present paper is concerned with the following aspects of this D → C change as studied by antigen-antibody precipitation in agar: the origin of the C-antigenic particles in heated suspensions, and their relationship to the D-antigenic particles initially present; the ability of certain agents, other than heat, to produce the change or modify its course; and, finally, the kinetics and possible mechanism of the reaction.

MATERIALS AND METHODS

Agar-diffusion tests.—The reactants and general procedures have been described in the earlier paper (Le Bouvier, 1959). The reactants most commonly used were 50-fold centrifuge concentrates of Type 1 (Brunhilde) virus grown in HuLi cells (Westwood, Macpherson and Titmuss, 1957), and either the Yale Brunhilde monkey antiserum or the "standard" Brunhilde rabbit antiserum. Types 2 and 3 polioviruses were represented by the Y-SK and Leon strains, respectively.

I am indebted for monkey antisera to Dr. E. M. Opton of the Section of Epidemiology and Preventive Medicine, Yale University School of Medicine; and to Drs. F. O. MacCallum and G. P. B. Boissard of the Virus Reference Laboratory, Colindale. Some of the rabbit antisera were kindly provided by my colleagues Drs. G. M. Churcher and G. Belyavin.

The tests were of the two-dimensional kind. Both antigen and antibody reactants were allowed to diffuse into the agar. In the usual pattern of experiment, the relative D or C antigen content of various preparations was determined with a single dilution (1/8) of the "standard" serum, the serum cup being surrounded by up to eight virus cups. Besides the "standard" serum, an "anti-C" serum was used to measure changes in the amount of C antigen. This serum, obtained from a rabbit immunized with Brunhilde virus heated at 60° for 20 min., gave no detectable precipitation with D antigen at a dilution of 1/8. Agar-diffusion tests were photographed after incubation for 3 days at 37°. From the position of the leading edges of precipitates, measured on the photographs, the relative antigen concentrations were calculated on the basis of the previous findings (Le Bouvier, 1959).

Infective-virus assay.—Virus dilutions were made in Earle's balanced salt solution containing 0·25 per cent lactalbumin hydrolysate and 5 per cent tryptic digest meat broth, adjusted to pH 7·0 with 2·78 per cent sodium carbonate. Plaque counts were done in 250 ml. Pyrex rubber-stoppered bottles. The HuLi cell line, derived from cultures of human embryonic liver (Westwood *et al.*, 1957), was grown as previously described (Le Bouvier, 1959). The agar-overlay procedure was adapted from that of Hsiung and Melnick (1957). Unwashed Difco-"Bacto" agar (2·4 per cent in distilled water) was mixed, in a 45° water-bath, with an equal volume of medium containing all the ingredients at double strength, so as to give the following final concentrations in the overlay: 0·25 per cent lactalbumin hydrolysate, 5 per cent tryptic digest meat broth, and 10 per cent inhibitor-free horse serum, in Earle's saline containing 0·11 per cent sodium carbonate. The mixture was not "gassed" with CO_2. Phenol red was omitted and neutral red added (1/60,000 in the final overlay mixture). Penicillin, 100 units/ml., and streptomycin, 100 μg/ml., were included.

Cultures were used as soon as they were confluent, *i.e.*, when the glass surface of area 23 cm² was covered by ~5 million cells. After removal of all the liquid medium, the virus inoculum of 0·5 ml. was distributed over the cell sheet and left in contact with it for 1 hr. at 37° before introduction of 10 ml. of the agar overlay. After 1 hr. at room temperature to permit firm gelling of the agar, the bottles were incubated at 37° with the cell sheet uppermost. Plaques were usually visible on the 2nd day and were well developed by the third. Counts were made during the period between the 3rd and 5th day.

Thermal inactivation of D antigen.—Heating was carried out in an adjustable water-bath with a temperature variation of not more than ± 0·2°. In the usual type of experiment, 0·05 ml. aliquots of virus concentrate were placed in 0·75-mm. thick, acid-cleaned, soda-glass tubes 7·5 cm. in length and of internal diameter 0·75 cm. The tubes, stoppered with rubber bungs, and with a volume of contained air of ~3 cm.³, were immersed for about 4/5 of their length in the water, and their contents agitated during the first 15 sec. of heating. Immersion for up to 20 min. produced no obvious condensation in the upper part of the tube. At the end of the period of heating, the tube was cooled in ice-water. Changes of volume and pressure during heating were not measured. Parallel tests using both stoppered and loosely capped

tubes showed no apparent difference in the inactivation rate of Brunhilde virus D antigen at 50°. However, when a larger volume of virus concentrate (0·5 ml.) was heated at 50° in a stoppered tube of this sort, and 0·05 ml. aliquots were removed at intervals, the inactivation rate was slowed to about one-half, despite thorough agitation of the contents.

Ultraviolet irradiation.—Brunhilde virus concentrate was irradiated in 1·5–2·5 ml. amounts in Petri dishes of diameter 9 cm. placed at a distance of 13·7 cm. from the source of ultraviolet light (UVL) and rocked at 50 cpm. Irradiation was done at room temperature (*c.* 20°). I am indebted to Dr. Elinor McCloy of the Department of Bacteriology, London School of Hygiene and Tropical Medicine, for the opportunity of using the UV lamp and for the information concerning dosage. The source of UVL was a 15-watt germicidal lamp emitting 95 per cent of its radiation in the wavelength 2537 Å. It had been calibrated by determining the rate of inactivation of coliphage T2 (Luria and Latarjet, 1947).

Other agents.—Poliovirus concentrates were tested in agar of varying pH, and subjected to freezing and thawing, drying, and treatment with mercuric chloride, merthiolate and phenol. The details are given in the section on Results.

RESULTS

Antigenic Conversion of Poliovirus Suspensions from D to C

Decrease of D antigen and increase of C antigen on heating

When poliovirus concentrates are heated at temperatures between 50° and 60°, their content of D antigen falls, while their C antigen concomitantly increases. Aliquots of a Brunhilde virus preparation were heated at 50° for varying lengths

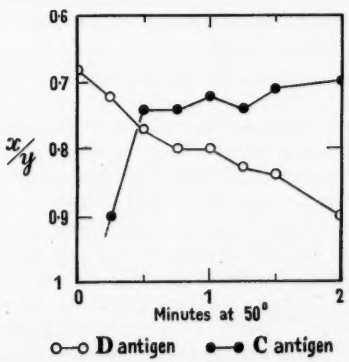

FIG. 1.—Decrease of Type 1 poliovirus D antigen and increase of C on heating at 50°
Ordinate { x = distance of leading edge of each precipitate from serum cup.
y = distance between serum and virus cups.

of time and then tested with single dilutions of 2 rabbit antisera, one containing mostly anti-D precipitins, the other anti-C. The results are shown in Fig. 1.

As the concentration of D antigen decreases, its precipitate is formed closer to the virus cup and the value for x_D/y increases. During the same period there is a rise in the concentration of C antigen. The reason for the flattening of the curve for the D antigen is not known. It is more usual, as will be seen later, for the points to fall on a straight line. The virus preparation used in this experiment consisted of a pool of three different Brunhilde virus concentrates, selected because they contained no detectable C antigen in the unheated state. The effect

seen may have been due to the presence of a relatively heat-resistant fraction in each of the three concentrates, or, more probably, to the greater resistance of one of them. Whatever the reason, the C antigen apparently increases *pari passu* with the decline of the D.

The relation between D-antigenic and C-antigenic particles

The concurrent increase in C and diminution in D antigen on heating could be coincidental, the D and C particles being independent of one another, but happening to be inactivated and activated, respectively, by equivalent degrees of heat. Alternatively, there might be a connexion between them. This could be an indirect one, the 2 kinds of particles being of separate origin. For example, " latent " precursors of C particles might depend for their activation upon the presence of some " unmasking " or inhibitor-destroying factor produced during heat inactivation of the D, or there might be a more direct connexion, C particles arising from the D particles themselves as the result of an antigenic change in their reactive surface.

That the formation of C antigen by heat is dependent on the presence of D is indicated by several consistent findings. C antigen fails to appear, or to increase in amount, in preparations containing no detectable D antigen before heating, whether the D-antigenic particles be separated by physical means, *e.g.* sucrose-density-gradient fractionation, or by specific aggregation with anti-D antibodies and removal of the precipitate by spinning. Moreover, the amount of C antigen formed on heating varies directly with the amount of D antigen initially present.

Experiments were made to try to distinguish between the possible origin of C particles from " masked " precursors in a manner only indirectly dependent on the D, and the alternative possibility that C-antigenic particles are formed directly from the D particles themselves.

Attempts to demonstrate an inhibitor of C-antigen precipitation gave negative results. Heated Type 1 virus concentrate was mixed in varying proportions with different lots of unheated homotypic or heterotypic concentrates. The amount of C precipitate formed by the heated virus was not reduced, even by those preparations which contained no " spontaneous " C antigen and which might have been expected to contain an excess of free inhibitor, if any such existed.

The possible presence of " masked " C-particle precursors was investigated by incubating Brunhilde virus concentrate, lacking detectable " spontaneous " C antigen, with an " anti-D " serum made by absorbing a Brunhilde monkey antiserum with the same virus heated at 60° for 20 min. The resulting D antigen-antibody aggregate was removed by spinning in the No. 40 Spinco rotor at 10,000 r.p.m. for 30 min. (mean RCF = 6,600 *g*). A control virus-diluent mixture was similarly treated. It was found that the capacity to form C antigen on heating was sedimented along with the D antigen-antibody aggregate from the virus-serum mixture ; whereas in the control mixture it remained in the supernatant. The C-particle precursor had either been specifically precipitated by the anti-D antibody, or been carried down mechanically with the D aggregate.

A similar experiment was done to test the effect of C antigen-antibody aggregation, as opposed to D. An " anti-C " serum was made by absorbing the Brunhilde monkey antiserum with unheated Brunhilde virus concentrate. This " anti-C " serum was mixed with heated Brunhilde virus in the presence of homologous

unheated concentrate containing no detectable "spontaneous" C antigen. Control suspensions consisted of the 2 virus preparations, either alone or combined, mixed with diluent instead of serum. The mixtures were spun at 10,000 r.p.m., and the supernatant fluids and deposits tested before and after heating. An increase of C antigen on heating was observed only in those mixtures in which unheated virus had been included: in each case it was seen only in the supernatant, and the amounts formed were comparable in test and control mixtures. There was therefore no reason to suspect sedimentation of a "masked" C-particle precursor as the result of mechanical entanglement or non-specific adsorption by the C antigen-antibody aggregate.

A direct conversion of D-antigenic particles into C-antigenic particles appears to be the simplest explanation of these findings.

Production of the D → C Change by Different Agents

Influence of pH on D and C precipitation

The solution used for preparing virus concentrates and for diluting all reactants was 0·14M sodium chloride buffered at pH 7·3 with 0·01 M phosphate. When exposed to the air, the pH of this weakly buffered solution and of the virus concentrates fell to about 7·1. On heating, the pH rose again to around 7·3 or higher (up to 7·6). To determine the effect of such variations on the precipitation reactions of poliovirus D and C antigens, tests were done in agar containing citrate-phosphate-borate buffer solutions of different pH (Theorell and Stenhagen, 1939). The pH of these solutions was determined by glass electrode. B.D.H. "Universal" indicator was added to the agar to detect any gross changes that might occur during the 3-days' incubation at 37°. The D and C antigen preparations consisted of 50-fold concentrates of the representative strain of each virus type, unheated or heated at 60° for 20 min., respectively. These were tested against selected single dilutions of monkey and rabbit antisera. The results are given in Table I.

TABLE I.—*Effect of pH on Poliovirus D and C Precipitation Reactions*

pH of agar	Poliovirus antigen preparations					
	Type 1		Type 2		Type 3	
	D	C	D	C	D	C
⩽3	−	−	−	−	−	−
4–5	+	±	±	−	+	−
6	+	+	+	−	+	−
7	+	+	+	+	+	+
8	+	+	+	+	± (→)	+
9–10	± (→)	+	tr. (→)	+	tr. (→)	+

D = the D antigen in unheated 50-fold virus concentrates.
C = the C antigen in the same concentrates heated at 60° for 20 min.
\+ = precipitate maximal, or nearly so, as judged by its density and distance from virus cup (see text).
± = precipitate about ½ to 1/4 of maximal amount.
tr. = trace of precipitate (< 1/4 of maximal amount).
− = no detectable precipitate.
(→) = increase in amount of C precipitate given by the unheated concentrate. (This is the only symbol which refers to the C-antigen content of *unheated* virus concentrates.)

Variation in the amount of precipitate was apparently related to the effect of pH changes on antigen rather than on antibody : the fainter the precipitate, the smaller was its distance from the virus cup.

Near the pH range of the heating experiments (pH 7·3–7·6), there appeared to be no significant alteration in the precipitation reactions of the representative strains of the 3 poliovirus types, except for the incipient reduction of the Type-3 D precipitation at around pH 8. With greater deviations from neutrality, however, a consistent pattern of impairment was observed with all the strains, the D activity being more sensitive to an alkaline, the C to an acid pH. This deleterious effect of low pH on the C precipitation was especially marked with the strains of Type 2 (Y-SK) and Type 3 (Leon). Strains of the same type may not behave exactly alike : in a few experiments with the MEF-1 strain of Type 2 virus, the D-antigen precipitation appeared to be as susceptible as the C to a pH in the region of 6. Below pH 3, the reactants in all cups appeared coagulated. In the pH range 9–10, the decrease in the amount of D precipitate given by the unheated concentrates of each type was accompanied by a rise in the C reactivity, suggesting a conversion of D antigen to C. The amount of precipitate formed by the heated concentrates remained unaffected by this alkaline pH. At higher pH levels (> 12) all poliovirus precipitating activity was abolished, C as well as D.

Concentrations of OH^- ions insufficient to effect the D $\rightarrow$ C change during 3 days at 37° will accelerate the reaction in Brunhilde virus at higher temperatures. Virus concentrate was mixed with equal volumes of veronal-buffer solutions of different pH (7·0–9·0) and heated at 50°. None of the mixtures showed any increase in C antigen before heating. But there was a progressive rise in the D $\rightarrow$ C conversion rate at 50° over this pH range : the D-antigen content was reduced to 1/10 in 3 min. at pH 7·0, and in 30 sec. at pH 9·0 ; the time for the control mixture with phosphate-saline diluent (pH 7·3) was $2\frac{1}{2}$ min.

D $\rightarrow$ C conversion of poliovirus by heat

Reproducibility of heating experiments.—The kinetics of the heat-inactivation reaction of Type-1 poliovirus D antigen are considered in more detail in a later section. As was mentioned earlier, in some cases the reaction rate may not be uniformly exponential (Fig. 1) ; but with most freshly made preparations the inactivation curve takes the form of a single straight line, as seen in Fig. 2. This shows the course of D-antigen inactivation at 50° in a suspension of Brunhilde virus. The virus was heated both as the undiluted 50-fold concentrate and as a 1 in 5 dilution in phosphate-buffered saline. Four replicate tests were made with each preparation. The points in Fig. 2 represent the individual readings. Straight lines were fitted by the method of least squares. An analysis of variance showed that the accord with linearity, for the 50-fold concentrate, was good within the limits of experimental error ($P > 0{\cdot}2$). There was no significant difference between the slopes of the two lines ($P > 0{\cdot}2$) ; but their intercepts on the ordinate were significantly different ($0{\cdot}01 > P > 0{\cdot}001$).

Variability of D $\rightarrow$ C conversion rate and its acceleration by horse serum.—With the majority of Brunhilde virus concentrates made by the method described, the D-antigen content was reduced to 1/10 after 1 to $1\frac{1}{2}$ min. at 50°. With occasional preparations, however, the time required might be as little as 1/4 min. or as much as 4 min. or even longer. Comparative tests of D-antigen inactivation rates were

always made on fractions derived from a given virus concentrate : these were heated simultaneously, where possible, or else within the space of a few hours. The inactivation rate is evidently affected by many factors which, even if known, may be difficult to standardize, particularly in crude concentrates of the kind used in this study. One example may be mentioned here : it illustrates the possible effect on the D → C conversion rate of the horse serum used in the culture medium.

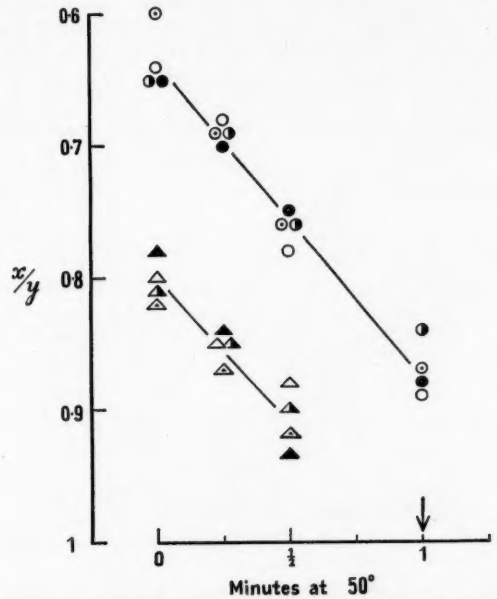

FIG. 2.—Replicate heat-inactivation tests of Type-1 D antigen

Ordinate { x = distance of leading edge of precipitate from serum cup.
y = distance between serum and virus cups.

Circles—50-fold virus concentrate
Triangles—1 in 5 dilution thereof } 4 replicate tests of each

Arrow indicates that leading edge of precipitate has entered virus cup

A batch of Brunhilde virus concentrate was divided into 2 lots, one of which was " washed " by two cycles of dilution in 10 vols. of phosphate-saline and sedimentation at 39,000 r.p.m. for 2 hr., the final pellet being resuspended in diluent to the original volume. The " unwashed " and " washed " concentrates were each divided into two portions, to one of which 1/10 volume of diluent was added, to the other 1/10 volume of unheated horse serum, as used in the culture medium. The 4 fractions were left for 1 hr. at room temperature (18°) and heated in parallel at 50°. The D-antigen inactivation slopes are shown in Fig. 3 : " washing " resulted in a slower loss of D antigen at 50° ; after addition of horse serum, the inactivation rates for both " washed " and " unwashed " virus were increased and became about equal. Heating the serum at 60° for 30 min. did not appear

to alter this effect. The degree of acceleration of the D → C change varied with the batch of horse serum.

Abolition of C reactivity at temperatures above 70°.—The antigenic change from D to C was produced by short exposure to temperatures at or just above 50°. D-antigen preparations of Types 2 and 3 tended, on heating, to be more rapidly changed than Type 1 preparations. But there were also marked divergences between strains of the same type, and indeed between different preparations of the same strain. In the range between 50° and 60°, poliovirus C antigen of Types 1 and 2 appeared relatively stable, that of Brunhilde virus showing little or no reduction even after heating at 60° for 2 hr. With Type 3 virus, however, there was usually some decrease in C antigen after 20 min. at 60°. At higher temperatures the C antigen of all 3 types, and with it all poliovirus precipitating activity, was lost. Differences between the representative strains of the 3 types were

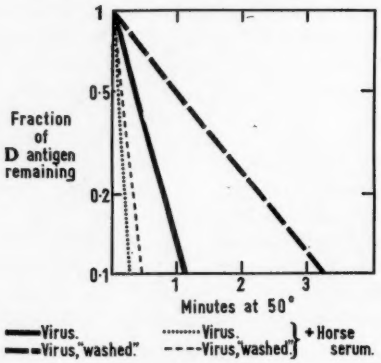

FIG. 3.—Acceleration of D-antigen inactivation in a Brunhilde virus concentrate by horse serum.

readily demonstrable, even with the rough test method employed. Aliquots of 50-fold virus concentrates were heated for 1-min. periods at various temperatures from 60°–100°. The temperatures which abolished all detectable C activity in this time were ~ 75° for Type 3, ~ 80° for Type 2, and ~ 85° for Type 1.

Infectivity loss and D → C change induced by heat and ultraviolet light

Heat and ultraviolet irradiation can both bring about the antigenic change from D to C. But whereas with heat the inactivation of virus infectivity and the decline of D antigen proceed at similar rates, with UV irradiation the abolition of infectivity is much the more rapid (Le Bouvier, 1955 ; Roizman *et al.*, 1959). Further evidence of this contrast is presented in Figs. 4 and 5. In one experiment, the loss of infectivity on heating occurred more rapidly, in another more slowly, than the change in the D antigen, with about a 2-fold difference in the reaction rates ; with UV irradiation, however, infectivity dropped to 10 per cent of its original level about 30 × more quickly than D reactivity. The findings are possibly related to differences in the mechanism of virus degradation and may suggest a link between the D → C change and the way in which the virus-particle infectivity is destroyed by heat as distinct from UV light. In Fig. 5 there is some suggestion

of a " multi-hit " type of inactivation curve for the D antigen. Its significance is not clear. With a different concentrate, irradiation of the D antigen produced a linear inactivation, and this was also the finding of Roizman, Mayer and Roane (1959).

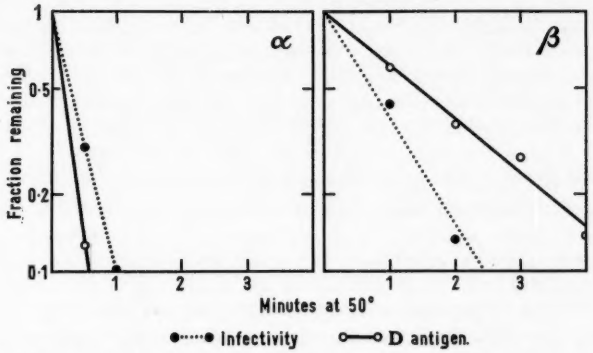

FIG. 4.—Infectivity loss and D-antigen inactivation induced in Brunhilde virus by heat

With the virus batch α, infectivity titrations were done before concentration (titre before heating = $10^{8\cdot1}$ plaque-forming units per ml.)

With β, infectivity titrations were done on the 50-fold concentrate (titre before heating = $10^{9\cdot9}$ plaque-forming units per ml.).

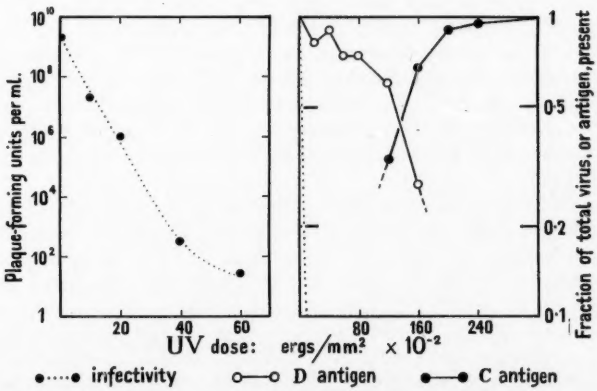

FIG. 5.—Infectivity loss and D → C change induced in Brunhilde virus by ultraviolet light. Infectivity titre of 25-fold virus concentrate before irradiation = $10^{9\cdot3}$ plaque-forming units per ml.

No C antigen detectable with UV dose $\leq$ 8,000 ergs/mm.².
No D antigen detectable with UV dose $\geq$ 20,000 ergs/mm.².

Treatment of 2 ml. of a 25-fold Brunhilde virus concentrate with a dose of UV irradiation of 600 ergs/mm.² resulted in a 90 per cent decrease in infectivity, whereas $\sim$ 20,000 ergs/mm.² were needed to effect a similar percentage reduction of the D antigen. As with heat, a more prolonged irradiation with UVL led to the loss of all C reactivity : in this preparation no C antigen was detectable after

a dose of 140,000 ergs/mm.^{2} To determine whether some third kind of specific poliovirus precipitinogen had been formed, which was unable to react with antibodies present in the " standard " or " anti-C " Type-1 rabbit sera, a rabbit was immunized with the same Brunhilde virus concentrate irradiated with a dose of 160,000 ergs/mm.^{2} Two intravenous injections of 1 ml. were given 3 weeks apart. Undiluted serum taken before, and 1 week after, the second injection contained small amounts of anti-C antibody, but gave no precipitate with either D antigen or the UV-irradiated concentrate used for immunization. The low levels of C precipitin were probably evoked by traces of C antigen remaining in the inoculum, even though it did not contain enough to form a detectable precipitate in agar.

Induction of the D → C change by agents other than heat, UVL and hydroxyl ions

This section deals briefly with some of the ways in which different physical and chemical agents may influence the poliovirus D → C conversion. In testing chemical compounds two methods were employed, of which examples are given below : incorporation of the substance in the agar ; and pre-incubation of virus (or serum) with the agent, usually for 1 hr. at 37°, before introduction of the mixture into the cups. In a few experiments the virus was spun down after incubation, and the pellet resuspended in diluent. Test plates were held at 37° or at 4°.

The process of D → C antigenic conversion was brought about, or modified, by a variety of agents and procedures. With some, the effect appeared to be independent of temperature : *e.g.*, lyophilization resembled drying at higher temperatures in causing the D → C change in concentrates of all 3 poliovirus types ; the dried virus in each case was resuspended in water at 4°.

Other agents apparently interacted with temperature in bringing about the D → C change. In sufficient concentration they accelerated the reaction, so that it could be observed at temperatures at which it was usually too slow to be practically measurable (4° to 37°). In somewhat lower concentrations they increased the rate of D → C change in virus heated at 50°. In contrast to these " accelerators ", some agents were found to retard the process, while others again appeared to be " neutral ".

Examples of D → C accelerating agents were mercuric chloride, merthiolate (sodium ethylmercurithiosalicylate) and phenol. These are considered below in more detail. The concentration of Brunhilde virus D antigen was reduced by exposure to acetone or ethanol for 5 hr. at 4°, or to 0·1 per cent potassium permanganate for 1½ hr. at 37° ; but these compounds were not sufficiently studied to establish a concomitant rise in C antigen. The action of horse serum has been mentioned in an earlier section.

The rate of D → C change in a Brunhilde virus concentrate at 52° was slowed if the virus was suspended in hypertonic solutions of sodium chloride. The degree of retardation increased progressively with the molarity : in 0·5 M solution the time taken for the D antigen to fall to 1/10 of its original concentration was about 10 × as long as in 0·14 M solution. At salt concentrations higher than 0·5 M, the rate of D → C change at this temperature was too slow to be conveniently measurable. Suggestive evidence was also obtained of retardation of the change in Brunhilde virus at 50° after treatment with formaldehyde at pH 7 (0·1 per cent for 5 weeks at 4°), or after incubation with 0·01 per cent L-cystine for 30 hr. at 37°, a procedure which has been found to stabilize the infectivity of a heat-sensitive line of Brunhilde virus (Pohjanpelto, 1958).

Exposure of Brunhilde virus to ether or chloroform for 5 hr. at 4° caused no detectable reduction in D antigen. The D → C conversion rates at 50° of virus so treated were not determined. The D-antigenic character of strains of each virus type was apparently not affected by freezing and thawing of virus concentrates up to 5 times, but this treatment caused aggregation of other, presumably non-viral, components, which could then be separated by spinning, leaving a clearer virus reactant. Pre-incubation of Brunhilde virus at 37° for 3 days (followed by the usual 3-day test at 37°) produced no detectable diminution in D antigen, nor did it lead to a more rapid D → C conversion at 50°. That some such effect might be expected, however, if the sojourn at 37° were further prolonged is suggested by the fact that 4 months' storage at 4°, though leaving the D-antigen concentration still unaltered, did result in an increased rate of change from D to C on heating, as compared with the same Brunhilde virus concentrate stored at −23°.

The results of incorporation tests with mercuric chloride and merthiolate, and of a pre-incubation test with phenol, are presented in Tables II and III. These and other agents causing detectable changes in the precipitation reaction evidently act largely, if not entirely, on the antigen; for diminution in the amount of precipitate is accompanied by a shift of the band towards the virus cup. Selected dilutions of monkey and rabbit antisera were used.

TABLE II.—*Effect of Mercuric Chloride and Merthiolate on Poliovirus D and C Precipitation Reactions*

Concentration of agent in agar (% w/v)	Poliovirus antigen preparations: Type 1 (Brunhilde) D	Type 1 (Brunhilde) C	Type 2 (MEF-1) D	Type 2 (MEF-1) C	Type 3 (Leon) D	Type 3 (Leon) C
Mercuric chloride:						
None	+	+	+	+	+	+
0·002	− (→)	+	− (→)	+	− (?→)	±
0·01	− (→)	+	−	tr.	−	−
Merthiolate:						
None	+	+	+	+	+	+
0·001	+	+	+	+	tr. (?→)	±
0·01	tr. (→)	+	− (→)	+	−	−
0·1	− (→)	+	− (→)	±	−	−

Test read after 3 days' incubation at 37°.
D = the D antigen in unheated 50-fold virus concentrates.
C = the C antigen in the same concentrates heated at 60° for 20 min.
\+ = precipitate maximal, or nearly so, as judged by its density and distance from virus cup (see text).
± = precipitate about ½ to 1/4 of maximal amount.
tr. = trace of precipitate (< 1/4 of maximal amount).
− = no detectable precipitate.
(→) = increase in amount of C precipitate given by the unheated concentrate. (This is the only symbol which refers to the C-antigen content of *unheated* virus concentrates.)

In the experiments with mercury compounds summarized in Table II the chelating agent versene (disodium dihydrogen ethylene-diamine-tetra-acetate) was left out of the agar. In another experiment it was incorporated in 0·07 per cent final concentration in the plate containing 0·01 per cent merthiolate. As a

result, the Type 1 and Type 2 strains gave maximal D precipitation (+) comparable with that seen in the control plates. With the Type 3 strain, however, the most sensitive to the action of merthiolate, this concentration of versene was not fully protective and the D precipitation was submaximal (±). The activity of versene in countering the D → C accelerant effect of merthiolate is presumably related to its function in safeguarding the immunizing potency of merthiolate-preserved poliovirus vaccine (Davisson *et al.*, 1956). Versene was also effective against the D-modifying action of mercuric chloride.

A plate containing 0·1 per cent merthiolate, but kept at 4° instead of 37°, showed no differences from the control beyond a slight reduction in the amount of D precipitate formed by the strains of Types 2 and 3.

TABLE III.—*Effect of Phenol on the Precipitation Reactions of Brunhilde Virus Antigens*

Concentration of phenol in virus-phenol mixture (% w/v)	Antigen preparation	
	D	C
None	+	+
0·5	+	+
1·0	±	+
1·5	tr. (→)	+
2·0	− (→)	+
2·5	−	tr.

Virus-phenol mixtures incubated at 37° for 1 hour before being placed in the cups.
Test read after 3 days at 37°.
Symbols mean the same as in Table II.

Table III shows the result of a test of Brunhilde virus 50-fold concentrate, unheated or heated at 60° for 20 min., of which aliquots were pre-incubated for 1 hr. at 37° with equal volumes of varying dilutions of phenol in phosphate-saline. The pH of the initial 5 per cent phenol dilution was adjusted to 7·1 with 1 N NaOH. Phenol concentrations below 1 per cent, which did not induce the D → C change at 37°, were nevertheless effective, in proportion to their strength, in hastening the conversion of Brunhilde virus at 50°. Treatment with water-saturated phenol at 4°, as in the extraction of virus ribonucleic acid, degraded both D and C antigens beyond recognition.

Kinetics of the D → C Change

The modification of poliovirus Type-1 D antigen by heat has some of the attributes of a unimolecular, or of a pseudo-unimolecular, reaction. The inactivation rate was found to be exponential, at least over the greater part of the measurable course of the reaction, and the slope of the inactivation curve was independent of the initial D-antigen concentration within the narrow range that could be tested (Fig. 2). This linear inactivation can also be seen in Fig. 6, which illustrates the marked temperature dependence of the D → C conversion.

Brunhilde virus 50-fold concentrate was heated at different temperatures between 48° and 52°, and the various reaction rate constants calculated from the equation $k' = 1/t$, where t is the time in minutes at which the fraction of D antigen left is 0·37, or e^{-1}. There was about a 2·7-fold increase in the value of k' for each

1-degree rise in temperature. A corresponding gradation, not shown in Fig. 6, was observed in the rate of emergence of C antigen at these different temperatures. Over the 4-degree span within which experiments were feasible, a linear relationship existed between the logarithm of the rate constants for D-antigen inactivation and the reciprocal of the absolute temperature (Fig. 7).

Although the evidence so far obtained suggests that the D → C change may be a first-order reaction with respect to concentration as well as time it must be admitted that the range both of temperature and of concentration in which it could be studied was exceedingly small. The preparations used in these experi-experiments were unpurified and no electron-microscopic particle counts were attempted. A rough estimate of the mass of virus present may be made, however, by assuming that there were between 50–500 non-infective particles for each one capable of producing a plaque, on the basis of the findings with polio-

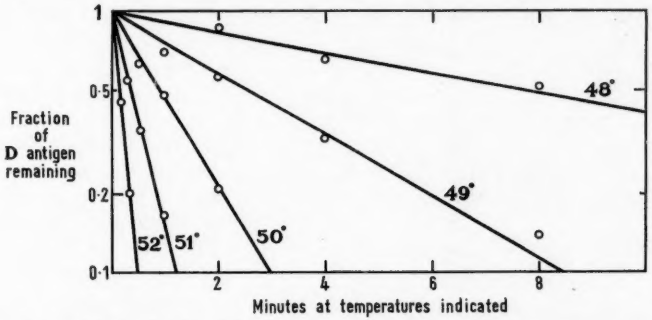

FIG. 6.—Inactivation of poliovirus Type-1 D antigen at different temperatures.

virus grown in monkey-kidney and human-amnion cells (Schwerdt and Schaffer 1955 ; Schwerdt and Fogh, 1957). The infectivity titre of Brunhilde virus liquids before concentration was usually about 10^8 plaque-forming units/ml. Taking the virus-particle mass as $1{\cdot}1 \times 10^{-17}$ gm. (Schaffer and Schwerdt, 1959), an average 50-fold Brunhilde virus concentrate would therefore be expected to contain $10^{11.4}$—$10^{12.4}$ particles/ml., or a virus mass of between 3 and 30 μg/ml.

There have been a number of reports of apparently first-order thermal inactivation reactions with different viruses (*e.g.*, Price, 1940 ; Lauffer, Price and Petre, 1949 ; Kraft and Pollard, 1954 ; Youngner, 1957 ; Kaplan, 1958 ; Luria, 1953 ; Pollard, 1953). For purposes of comparison, therefore, thermodynamic constants were calculated for the D → C change in poliovirus : $\Delta H^{\ddagger}$ (taken as $\simeq E_{\text{exp.}}$, the experimental activation energy, derived from the data in Fig. 7) was found to be 203 kcal./mole, and $\Delta S^{\ddagger}$ to be + 570 entropy units. Whatever the exact significance of these values may be, the activation of D particles during the antigenic conversion of poliovirus suspensions from D to C appears to be associated with a large total heat energy change and a correspondingly large increase in entropy.

DISCUSSION

By physical means, poliovirus suspensions of a given strain can often be divided into fractions which react predominantly either as D or as C antigens in direct

tests of complement fixation or precipitation in agar (Mayer *et al.*, 1957 ; Le Bouvier *et al.*, 1957 ; Roizman *et al.*, 1958, 1959). It has also been possible to separate the two kinds of antigenic particles by specific absorption, at least from certain sorts of virus suspension and using selected animal antisera (Hummeler and Hamparian, 1958 ; Le Bouvier, 1959). Most of the particles in such absorbed preparations possess, in effect, either D reactivity, or C, but not both at the same time.

When suspensions containing D antigen are heated, C antigen appears, or, if already present, increases in amount. The present experiments indicate that the precursors of C-antigenic particles are either the D particles themselves, or particles that have so far proved to be indistinguishable from them. In the absence of contrary evidence, there seems little reason to consider these precursors

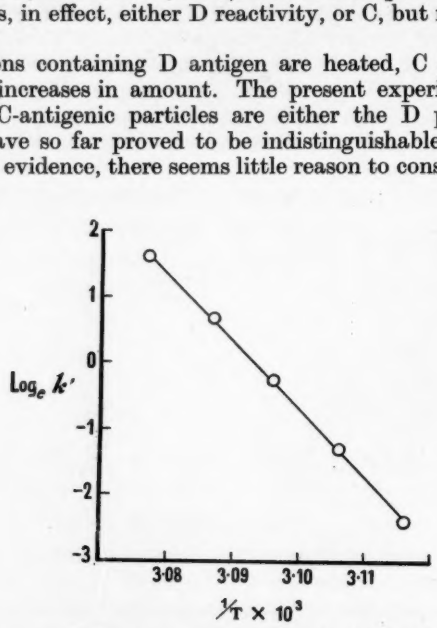

FIG. 7.—Arrhenius plot constructed from data shown in Fig. 6.
k' = inactivation rate constant of D antigen at absolute temperature T.

as " masked " C particles, or as complexes of C particles with some inhibitor. All the results so far obtained are consistent with the hypothesis that particles with D reactivity are themselves changed by heat into C-antigenic particles.

The D → C change has the form of a unimolecular, or of a pseudo-unimolecular, reaction. In most Brunhilde virus 50-fold concentrates the change is measurable in about 90–95 per cent of the particles. In this majority of particles the D-antigen inactivation curve is usually exponential. The rate-determining step in the reaction could be the breaking of a secondary hydrogen bond, such as may occur in certain protein denaturations (Laidler, 1958), or an oxidation, as has been suggested by Pohjanpelto (1958) for the heat inactivation of poliovirus infectivity. Whatever the nature of this reaction may be, it can evidently be precipitated by a number of factors. These presumably work in different ways to produce the same result—an altered architecture of the " shell " of protein sub-units which constitutes the external layer of the D-antigenic particle (Finch and Klug, 1959). Heat and ultraviolet light might be expected to act by disrupting interhelical linkages ; phenol, by taking the place of amino groups in forming hydrogen bonds with

carbonyl groups; an increase in OH^- ion concentration, by causing ionization changes; mercuric and ethylmercuric ions, as also the foregoing agents, by severing disulphide bonds. Lyophilization, where the effect is probably due to oxidation during the drying, has been shown to have a strong splitting action: the virus particles, after resuspension in water, are found to have dissociated into fragments (Hampton, 1955; Ehrenberg and Polson, 1958).

As to the way in which the change in antigenic surface would be determined by the antecedent chemical events, various possible mechanisms can be imagined. There is at present no balance of evidence in favour of any particular one. Perhaps the simplest supposition would be that the critical reaction makes irreversible a change in the structure or arrangement of protein helices which can be initiated by several different means. Any proposed explanation of the antigenic differences between D and C particles would have to take into account the observed conjunction of deficient ribonucleic acid (RNA), altered particle structure, and C reactivity (Schwerdt, 1957; Le Bouvier *et al.*, 1957) and would depend on the answers to the following questions, among others: Can virus particles be antigenically D though lacking nucleic acid, a possibility suggested by the finding of non-infective, relatively slowly sedimenting, D-antigenic particles on density-gradient fractionation of purified virus (Roizman *et al.*, 1958)? Conversely, do particles ever acquire the C-antigenic character while retaining their RNA? Are the sub-units, and groups of sub-units, from which the poliovirus D particle is built up, themselves antigenically C? If so, do they require the presence of virus RNA to be assembled into particles of D-antigenic configuration?

SUMMARY

The antigenic character of poliovirus suspensions can be modified by heat, a process referred to as the D → C change. The nature and kinetics of this change have been studied by specific precipitation in agar, using centrifuge concentrates of tissue-culture virus and monkey or rabbit antisera.

The D → C change in the reactivity of a poliovirus suspension most probably reflects a conversion of the virus particles from the D-antigenic form to the C. This change is produced by short exposure to temperatures between 50° and 60°. Between 75° and 90°, depending on the strain of virus, the C-antigenic activity is rapidly destroyed. The D → C conversion of poliovirus can be brought about at lower temperatures (4°–37°) by a variety of agents, including ultraviolet light, drying, mercury compounds, phenol and an alkaline pH.

The heat modification of poliovirus Type-1 D antigen appears to be a unimolecular, or a pseudo-unimolecular, reaction, in which activation of the D particles is associated with a large heat energy change and a corresponding increase in entropy. It is suggested that various factors may promote the D → C change by causing a distortion of the protein structure and facilitating an irreversible reaction in the D particle.

I am much indebted to Professor Wilson Smith for his interest, advice and criticism; also to Dr. G. Belyavin and Dr. G. M. Churcher of this department, Dr. E. W. McCloy of the Department of Bacteriology, London School of Hygiene and Tropical Medicine, and Dr. A. J. Parker of the Department of Organic Chemistry, University College, for their help and suggestions.

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SURVEY OF PAPERS

TAYLOR-ROBINSON and RONDLE, using the agar gel-diffusion precipitation technique, showed that lines between chickenpox and zoster antigens (vesicle fluids or concentrated tissue culture fluids) and undiluted or concentrated convalescent sera from patients with either of these diseases, were continuous. Three lines were discernible in the system. Usually sera with the highest complement fixation titres gave the sharpest lines of precipitate, but precipitation was a less sensitive test than complement fixation. The results support the conclusions drawn from their other work, that the agents of chickenpox and zoster are identical (p. 517).

TAYLOR-ROBINSON, using cultures of human amnion cells and fibroblasts, did neutralisation tests against zoster and chickenpox vesical fluids and suspensions of cells infected with these viruses on acute and convalescent sera from cases of both diseases. He found the agents of the two diseases immunologically indistinguishable ; and that neutralising antibody in contrast to complement fixing antibody persisted for many years in the sera of patients who had had chickenpox (p. 521).

MIMS injected massive doses of ectromelia virus intravenously in mice and titrated homogenised organs at intervals of time thereafter. He also searched material for virus antigen by fluorescence microscopy. He found that 95 per cent of input was rapidly taken up by cells lining the liver sinusoids. Less than 1 per cent remained in circulation, and that was attached to leucocytes and platelets (p. 533).

MIMS again used fluorescence microscopy and infectivity titrations to follow the growth of ectromelia virus in the livers of intravenously inoculated mice. Sections of infected liver from mice previously inoculated with Indian ink were stained with fluorescent antibody and examined by ultra-violet light and phase contrast successively. Ectromelia virus antigen was seen to be confined to cells lining sinusoids (labelled by their content of ink) for 9 hr. after infection. From 10 hr. onward increasing fluorescence was seen in hepatic parenchymal cells (p. 543).

GRANT found that dioxan inhibits antigen antibody precipitation. He has studied its effect upon 5 precipitin reactions in which the antisera were from rabbits immunized with protein antigens. He found that the speed of the reactions was slowed and that the amount of precipitate was decreased in each case. Treatment with dioxan did not denature the antigens (p. 551).

VAN TRIET has developed a process for the enzymatic purification of diphtheria or tetanus antitoxin obtained from sheep. He describes experiments that led to the final process whereby high titre antitoxins were produced that contained relatively little protein. (p. 559)

SCOTT and SOLIS occluded the portal veins of rabbits to investigate the formation and embolization of thrombi induced by injection of a convertin-rich (Factor VII) serum. The amount of thrombus formed was broadly proportional to the circulating concentration of convertin. In addition the formation of thrombi, when associated with a high level of fibrinolysin, resulted in increased friability of the clot, thus predisposing to embolization (p. 567).

TURK has continued his study of substances in serum resembling antibody. Fractions of normal human serum, obtained by treatment with cold ethanol, were titrated for immune adherence activity against some bacterial antigens and starch, and for agglutinins of Group

A erythrocytes. The author concludes that immune adherence activity lies in a fraction of the β globulin (p. 578).

HOLBOROW, PATRICIA BROWN, ROITT and DEBORAH DONIACH used sera from patients with chronic thyroiditis to treat sections of normal human and other mammalian thyroid glands. Fluorescein-conjugated rabbit anti-human-globulin serum revealed fixation of globulin in the sections. Sera containing thyroglobulin precipitins caused staining of follicular colloid. Sera fixing complement with thyroid "microsomal" antigen caused staining of the cytoplasm of acinar cells. The specificity of these reactions was demonstrated with suitably absorbed sera (p. 583).

HARRIS subjected young male guinea-pigs to 150–170 r whole body irradiation. The neutrophils in the bone marrow and in the peripheral blood were studied by repeated counts. The damage done to the neutrophils by irradiation was followed by recovery in two separate phases; the first phase occurred between the fourth and twelfth day but was not maintained, while the second phase on about the fourteenth day resulted in complete recovery. It was found that the cytological changes in the marrow were reflected in the peripheral blood after an interval of about 4 days (p. 589).

NESTEL investigated the hypothesis that the fat in atheromatous plaques is derived from disintegrating red blood corpuscles. Accordingly an attempt was made to incorporate thrombi with and without erythrocytes in an arterial wall and to compare the fat content of the resulting lesions. Injections of erythrocyte-containing and red cell free clots into the ear veins produced lesions in the pulmonary arteries of male rabbits. The mural thrombosis produced by both types of clots contained fat, but in every area of mural thrombosis haemosiderin or red cells were present. The author concludes that red blood corpuscles form one source of the fat in atheroma without excluding the possibility of other lipoid sources. (p. 601).

LE BOUVIER has used agar-diffusion tests and infectivity titrations by a plaque technique to study the antigenic change D $\rightarrow$ C in poliovirus particles. This change is made by heat and hastened or retarded by other physical and chemical agents. The author suggests that factors promoting the change do so by distortion of the protein structure of the D particle, facilitating an irreversible reaction therein (p. 605).

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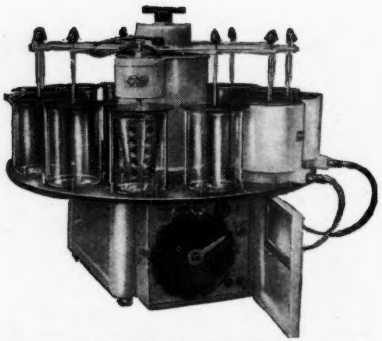

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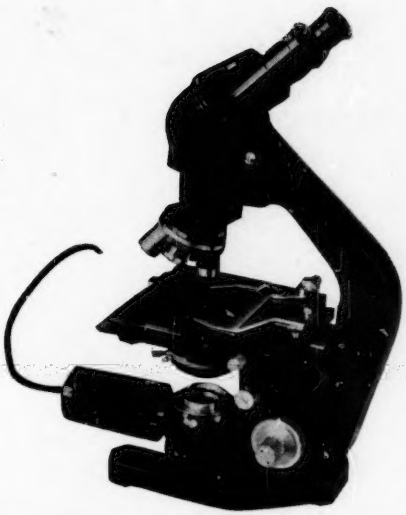

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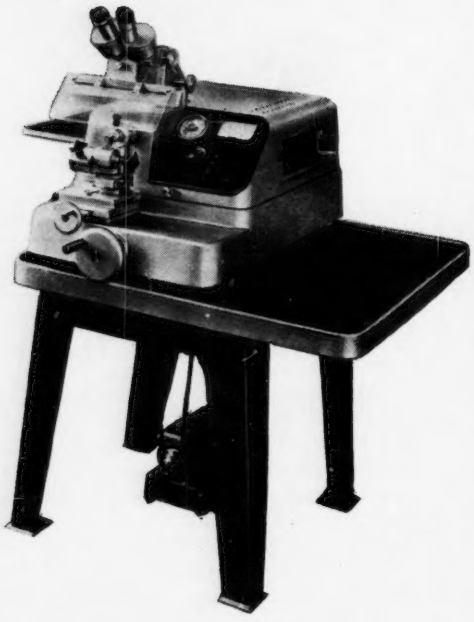

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